Pergamon

Bioorganic & Medicinal Chemistry, Vol. 2, No. 7, pp. 631-637, 1994

0968-0896/94 $7.00 + .00

0968-0896(94)00061-1

Enzymatic Synthesis of Isotopically Labeled Isoprenoid Diphosphates

Dale J. Christensen[§] and C. Dale Poulter*

University of Utah, Department of Chemistry, Salt Lake City, UT 84112, U.S.A.

Abstract—Recombinant yeast isopentenyl diphosphate (IPP) isomerase and avian farnesyl diphosphate (FPP) synthase from overproducing strains of *Escherichia coli* were used to synthesize FPP from IPP and dimethylallyl diphosphate (DMAPP). [2,4,5-$^{13}C_3$]IPP and [2,4,5-$^{13}C_3$]DMAPP were synthesized from ethyl [2-^{13}C]bromoacetate and [1,3-$^{13}C_2$]acetone. These compounds were used as substrates for enzymatic synthesis of FPP selectively labeled at the first or third isoprene residue or at all three.

Introduction

In recent years, enzymes have been used with increasing frequency in organic chemistry. Some common applications involve lipase or acylase catalyzed resolutions of enantiomeric mixtures of amines or alcohols[1] and stereoselective oxidations and reductions by dehydrogenases.[2] Less commonly, enzymes in a biosynthetic pathway are used to construct complex natural products from simpler precursors.[3] This approach sometimes can be used to construct molecules with a high degree of regio- and stereospecific control from smaller and more synthetically accessible compounds.

During the early stages of the isoprenoid pathway, isopentenyl diphosphate (IPP, **1**)[4] is isomerized to dimethylallyl diphosphate (DMAPP, **2**).[5] DMAPP then serves as the electrophilic primer for the production of all other isoprenoid diphosphates, including geranyl diphosphate (GPP, **3**), farnesyl diphosphate (FPP, **4**), and geranylgeranyl diphosphate (GGPP, **5**).[6] These compounds are converted into over 23,000 different natural products. Some representative examples include: steroids, used in eukaryotic membranes and as hormones in animals;[7] prenylated proteins, components in signal transduction pathways;[8] carotenoids, used as pigments and photoreceptors in photosynthetic and visual systems;[9] cytokinins, used as plant hormones;[10] glyceryl ethers, used as the major components of archaebacterial membranes;[11] mono- and sesquiterpenes, used for protection and as hormones in plants;[12,13] and dolichols, used as sugar carriers for glycoprotein biosynthesis in eukaryotes and for synthesis of bacterial cell walls.[14] As illustrated in Scheme I, the isoprene moieties in all of these metabolites are derived from IPP.

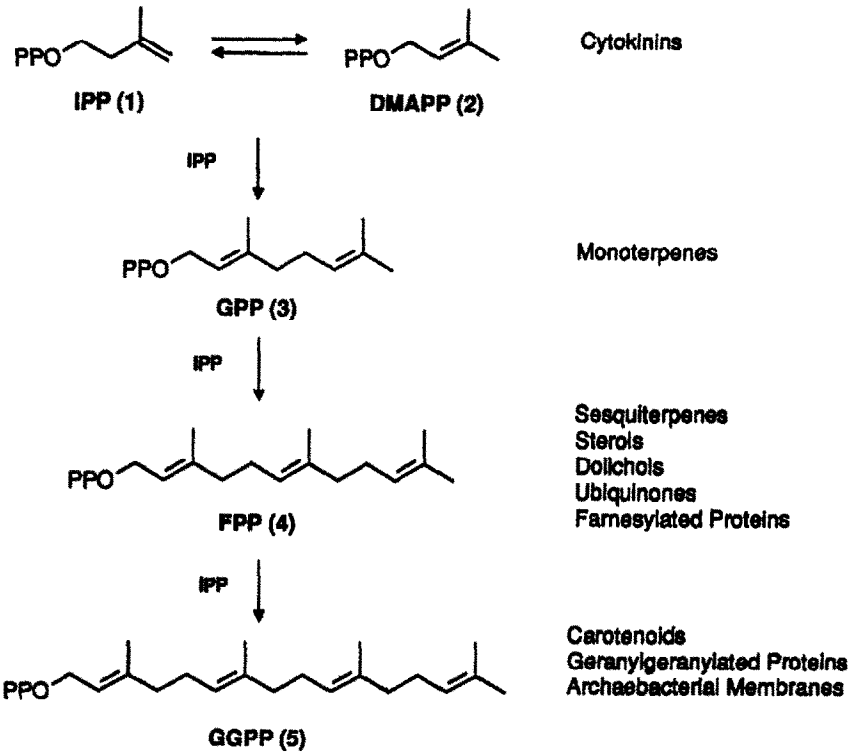
Many of the pioneering advances in the use of isotopes to study biosynthetic pathways were made with isoprenoid compounds. Some prominent examples include deuterium and tritium labeling to deduce stereochemistry at methylene and methyl groups[15,16] and applications of carbon NMR spectroscopy to elucidate patterns of incorporation.[17] These experiments depend on having a source of appropriately labeled materials. As part of our investigation of the biosynthesis of isoprenoids, we have recently cloned and expressed the genes for several enzymes in the pathway.[18,19] We now report a simple procedure for labeling of IPP and DMAPP and incorporation of the labeled isoprene units into FPP using recombinant IPP isomerase and FPP synthase.

Results and Discussion

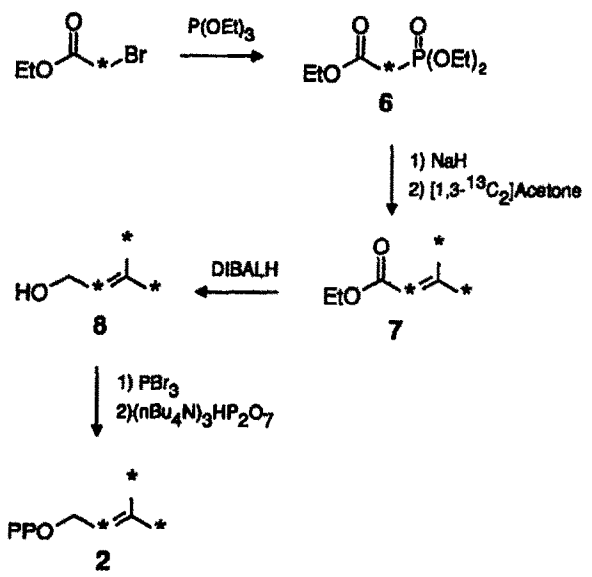
Isotopically labeled compounds are important tools for studying biosynthetic pathways. In the isoprenoid pathway, few intermediates are available in labeled, or for that matter in unlabeled, form from commercial sources. Typically, isoprenoid diphosphates used in biosynthetic experiments are synthesized from the corresponding alcohols.[20] While it is relatively straightforward to introduce isotopic labels for the hydrogens at C(1), labeling other hydrogens or any of the carbons requires a substantial effort in organic synthesis. Recently, we constructed strains of *Escherichia coli* that produce recombinant forms of yeast IPP isomerase[21] and avian FPP synthase.[22] These two enzymes direct synthesis of FPP from IPP and can be used in a variety of combinations of enzyme and substrate to selectively label FPP from simpler, easily synthesized, five-carbon precursors. Several examples are presented to illustrate this concept.

Syntheses of IPP and DMAPP with ^{13}C at C(2), C(4), and C(5) are shown in Schemes II and III. The ^{13}C isotope for C(2) was derived from ethyl [2-^{13}C]bromoacetate. The labeled ester was converted to phosphonate **6** by treatment with triethyl phosphite,[23] and the resulting phosphonate was condensed with [1,3-$^{13}C_2$]acetone by a Horner–Emmons coupling[24] to give triply labeled ester **7**. [2,4,5-$^{13}C_3$]Dimethylallyl alcohol (**8**) was obtained in high yield

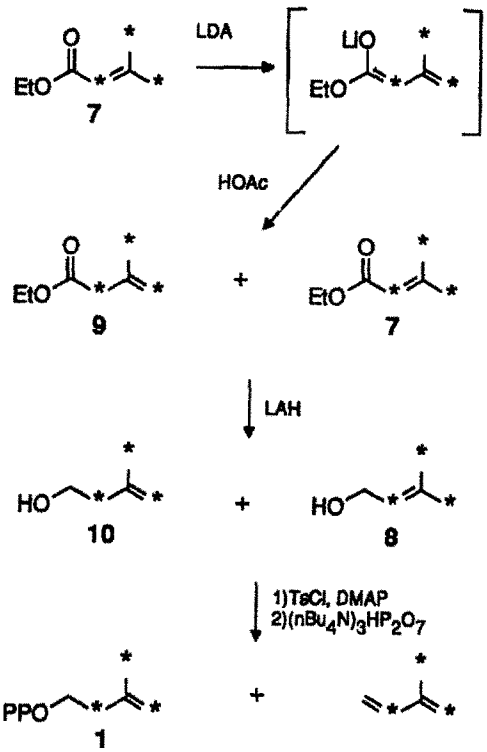
[§]Present address: Texas A&M University, Department of Chemistry, College Station, TX 77845, U.S.A.



Scheme I. Biosynthetic pathway for linear isoprenoid diphosphates.



Scheme II. Synthesis of [2,4,5-$^{13}C_3$]dimethylallyl diphosphate.



Scheme III. Synthesis of [2,4,5-$^{13}C_3$]isopentenyl diphosphate.

by reduction of **7** with DIBALH.[25] The allylic alcohol was converted to dimethylallyl bromide upon treatment with PBr_3, and the bromide was immediately treated with *tris*-tetra-*n*-butylammonium pyrophosphate to give [2,4,5-$^{13}C_3$]DMAPP. The diphosphate was purified by HPLC[26] and stored as a powder at −70 °C.

[2,4,5-$^{13}C_3$]IPP was also obtained from **7**. The α,β-unsaturated ester was deconjugated by formation of the extended enolate with LDA, followed by a kinetic quench at −78 °C with acetic acid in THF. This procedure gave a 15:85 mixture of α,β-**7** and β,γ-**9**, respectively. The isomeric esters and the alcohols obtained by LAH reduction were not easily separated. However, the allylic isomer was selectively destroyed in the next step. Treatment of the mixture with tosyl chloride in DMAP converted both alcohols to the corresponding tosylates. The homoallylic isopentenyl tosylate was stable to the reaction conditions,

whereas dimethylallyl tosylate decomposed to isoprene, which was easily removed during work-up. [2,4,5-$^{13}C_3$]IPP was obtained by treatment of isopentenyl tosylate with *tris*-tetra-*n*-butylammonium pyrophosphate, and the diphosphate ester was purified by HPLC.

The reactions shown in Schemes II and III can also be used to label IPP and DMAPP at C(1), using ethyl [1-^{13}C]bromoacetate, or C(3) using [2-^{13}C]acetone. Relatively simple procedures are also available that can be used to specifically label C(4) of IPP[27] or the *E* or *Z*-methyls of DMAPP.[28]

Yeast IPP isomerase and avian FPP synthase were obtained from *E. coli* transformants containing plasmids derived from pARC306N, an expression vector with an *E. coli* recA promotor and a phage T7 gene 10 ribosomal binding site.[21] The cytosolic proteins in *E. coli* strains JM101/pIPS241[21] and JM101/pMJY9-11[22] contained 30–40 % of IPP isomerase and FPP synthase, respectively, when grown in LB at 37 °C. The enzymes used in this study were partially purified by ion exchange chromatography on DE-52 cellulose, although supernatants from centrifugation of disrupted cells are sufficiently pure for most applications.

Incubation of [2,4,5-$^{13}C_3$]IPP with IPP isomerase and FPP synthase (1 unit of isomerase/50 units of synthase) labeled all three isoprene units to give [2,4,6,-8,10,12,13,14,15-$^{13}C_9$]FPP. As the reaction proceeded, the magnesium salt of newly formed FPP formed a white precipitate. Upon completion, the suspension was clarified by centrifugation. The pellet was solubilized by cation exchange with Chelex-100 in NH_4HCO_3 buffer, and labeled FPP was purified by reversed-phase HPLC. A ^{13}C NMR spectrum of this material (see Figure 1b) had all of the resonances seen for a sample of unlabeled FPP (Figure 1a) except for the peaks for C(1) at 66 ppm, C(5) and C(9) at 32 ppm, and the quaternary olefinic carbons near 140 ppm. The first isoprene unit in FPP was selectively labeled by incubation of [2,4,5-$^{13}C_3$]IPP and GPP with FPP synthase. In this case, a ^{13}C NMR spectrum of the product (see Figure 1c) had resonances at 21 ppm for the C(13) methyl, at 44 ppm for the C(4) methylene, and at 125 ppm for the C(2) olefinic carbon. In a similar manner, incubation of IPP and [2,4,5-$^{13}C_3$]DMAPP with FPP synthase gave FPP labeled in the third isoprene unit. The ^{13}C NMR spectrum of this material (see Figure 1d) had resonances at 22 ppm for the C(12) methyl, at 29 ppm for the C(15) methyl, and at 129 ppm for the C(10) olefinic carbon. The three incubations gave 10–12 mg of purified ^{13}C-labeled FPP in yields of *ca* 90 %.

Various combinations of labeled isoprene units in FPP can readily be obtained by judicious selection of labeled precursors and enzymes. For example, incubation of either IPP or DMAPP with IPP isomerase and FPP synthase uniformly incorporates the isoprene unit into all three positions. More selective incorporation is possible by using various combinations of IPP, DMAPP and GPP

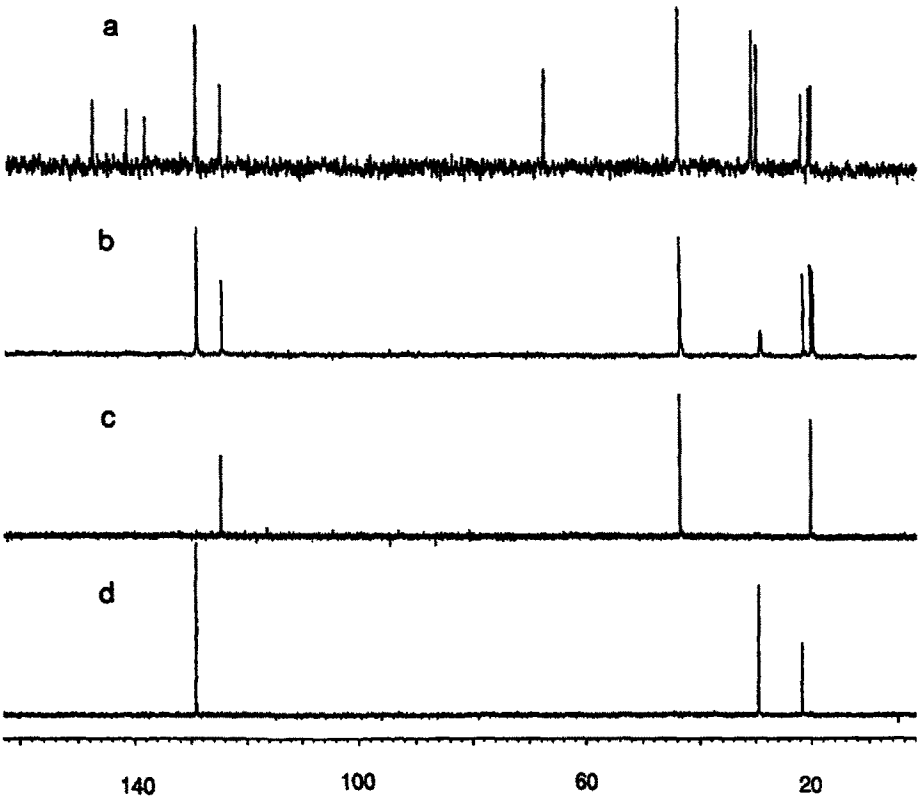


Figure 1. ^{13}C NMR spectra of FPP. (a) Unlabeled FPP at natural abundance; (b) [2,4,6,8,10,12,13,14,15-$^{13}C_9$]FPP; (c) [2,4,13-$^{13}C_3$]FPP; (d) [10,12,15-$^{13}C_3$]FPP.

with FPP synthase. Incubation of labeled IPP and GPP selectively labels the first isoprene unit, while incubation of IPP and labeled DMAPP selectively labels the last. The first two isoprene units are labeled when labeled IPP is condensed with DMAPP. At this point, there is no convenient method for selectively labeling the second isoprene in FPP because the reaction cannot be stopped after the first condensation. However, Blanchard and Karst[29] recently reported a yeast mutant that excretes geraniol, presumably because of an altered substrate selectivity for FPP synthase that inhibits the $C_{10} \rightarrow C_{15}$ chain elongation step. Thus, it may be possible to construct an FPP synthase variant by site-directed mutagenesis that synthesizes GPP which can then be elongated to FPP by wild-type enzymes.

Conclusion

FPP can be synthesized enzymatically from IPP and DMAPP or GPP using recombinant enzymes isolated from overproducing strains of *E. coli*. The enzymes can be readily purified by ion exchange chromatography and stored for several weeks prior to use. With the proper combination of substrates and enzymes, it is possible to use labeled derivatives of IPP and DMAPP to selectively introduce isotopes into first or third isoprene residues of FPP or to label all three. We have used these procedures to synthesize both ^{13}C-labeled and radiolabeled materials. The techniques should also be useful for both regio- and stereoselective incorporation of deuterium into the isoprene units.

Experimental

Synthesis of ^{13}C labeled IPP and DMAPP

General. All experiments were run in distilled solvents and oven dried flasks under a nitrogen atmosphere. Silica gel flash chromatography was performed with grade 60, 235–400 mesh silica gel (J. T. Baker). TLC was performed on silica gel 60 F-254 glass plates (EM Science) and visualized by UV light iodine, or dipping in 5 % phosphomolybdic acid in ethanol followed by heating. ^1H and ^{13}C NMR spectra were obtained in $CDCl_3$ or D_2O. Chemical shifts are reported in ppm downfield from internal TMS or DSS. ^{31}P NMR chemical shifts are reported relative to external 85 % H_3PO_4. Reagent grade THF was predistilled from CaH_2 and finally distilled from Na/benzophenone ketal. Reagent grade CH_2Cl_2 was distilled from CaH_2 and all other solvents were glass distilled prior to use. Unlabeled IPP, DMAPP, GPP and FPP were prepared by previously reported methods.[19] Enzyme activity was determined by the acid lability assay.[26] [2-^{13}C]Ethylbromoacetate was purchased from Isotec Inc., and [1,3-$^{13}C_2$]acetone was purchased from Cambridge Isotopes. Microcrystalline DE-52 cellulose ion exchange cellulose was from Whatman. All other chemicals were purchased from Aldrich or Sigma. Centriprep-10™ concentrators were purchased from Amicon.

Triethyl [2-^{13}C]phosphonoacetate (6). To 3.51 g (21 mmol) of ethyl [2-^{13}C]bromoacetate was added 4.98 g (30 mmol) of triethylphosphite. The mixture was heated at 60 °C with stirring. After 24 h, the mixture was allowed to cool to room temperature and placed under high vacuum (0.5 mm Hg) for 24 h to give 4.00 g (85 %) of a colorless oil; ^1H NMR (300 MHz) δ 1.29 (3H, t, J_{HH} = 7.2 Hz), 1.35 (6H, td, J_{HH} = 7.1 Hz), 2.97 (2H, dd, J_{CH} = 129.9 Hz, J_{PH} = 21.5 Hz), 4.19 (4H, qd, J_{HH} = 7.1 Hz, J_{PH} = 0.9 Hz), 4.20 (2H, q, J_{HH} = 7.1 Hz); ^{13}C NMR (75 MHz, ^1H decoupled) δ 14.11, 16.34 (d, J_{PC} = 6.2 Hz), 34.79 (d, J_{PC} = 133.6 Hz), 61.56, 62.68 (d, J_{PC} = 6.2 Hz), 165.27 (d, J_{PC} = 58.5 Hz). ^{31}P NMR (121 MHz) δ 20.44 (dt, J_{PC} = 133.6 Hz, J_{PH} = 21.5 Hz); High Resolution (HR)MS (CH_4, CI): $^{12}C_7{}^{13}C_1H_{18}O_5P$, calculated 226.0925, found 226.0934.

Ethyl [2,4,5-$^{13}C_3$]3-methyl-2-butenoate (7). To a suspension of 0.72 g (30 mmol) of NaH in 30 mL of THF in an ice-bath was added 4.00 g (18 mmol) of **6**, dropwise over several minutes. The suspension was allowed to warm to room temperature with stirring for 30 min and then cooled in an ice-bath. To the resulting solution was added 1.50 g (25 mmol) of [1,3-$^{13}C_2$]acetone, and the solution was allowed to warm to room temperature. After stirring for 18 h, 30 mL of 0.5 N HCl was added. The layers were separated, and the aqueous phase extracted with diethyl ether. The combined organic extracts were washed with saturated $NaHCO_3$, water and saturated NaCl, dried over $MgSO_4$, and concentrated by rotary evaporation to afford 1.42 g (61 %) of a colorless oil; ^1H NMR (300 MHz) δ 1.27 (3H, t, J_{HH}=7.2 Hz), 1.89 (3H, d, J_{CH} = 127 Hz), 2.17 (3H, d, J_{CH} = 129 Hz), 4.14 (2H, q, J_{HH}=7.2 Hz), 5.68 (1H, d, J_{CH} = 160 Hz); ^{13}C NMR (75 MHz, ^1H decoupled) δ 14.36, 20.16 (d, J_{CC} = 3.8 Hz), 27.35 (dd, J_{CC} = 3.8 Hz, J_{CC} = 3.8 Hz), 59.42, 116.18 (d, J_{CC} = 3.8 Hz), 163.95 (m), 166.71 (d, J_{CC} = 76.7 Hz); HRMS (EI): $^{12}C_4{}^{13}C_3H_{12}O_2$ calculated 131.0939, found 131.0953.

[2,4,5-$^{13}C_3$]3-Methyl-2-buten-1-ol (8). To a solution of **7** (0.67 g, 5.1 mmol) in 10 mL of CH_2Cl_2 at –70 °C, was added 13.0 mL (13 mmol, 1.0 M in CH_2Cl_2) of diisobutylaluminum hydride dropwise over a period of 10 min. After 2 h, 2 mL of CH_3OH was added, the solution was allowed to warm to room temperature, and 20 mL of saturated sodium potassium tartrate was added. The emulsion was stirred rapidly until the phases separated. The organic layer was removed, the aqueous layer extracted with CH_2Cl_2. The combined organic fractions were washed with brine, dried over $MgSO_4$, and concentrated by rotary evaporation to yield 0.45 g (99 %) of a colorless oil; ^1H NMR (300 MHz) δ 1.57 (1H, s, OH), 1.69 (3H, s), 1.75 (3H, s), 4.13 (2H, d, J_{HH}=6.9 Hz), 5.41 (1H, t, J_{HH}=6.9 Hz); ^{13}C NMR (75 MHz) δ 17.8 (d, $^2J_{CC}$ = 3.8 Hz), 18.0 (d, $^2J_{CC}$ = 3.8 Hz), 59.1 (d, $^1J_{CC}$ = 37.2), 124.3 (d, $^2J_{CC}$ = 3.8 Hz), 135.8 (m); HRMS(EI) $^{12}C_2{}^{13}C_3H_{10}O$ calculated 89.0821, found 89.0824.

[2,4,5-$^{13}C_3$]3-Methyl-2-butenyl diphosphate (2). To a solution of **8** (0.258 g, 2.9 mmol) in 5 mL pentane at 0

°C, was added 0.955 g (3.5 mmol) of PBr_3. The solution was stirred for 20 min, at which time 1.0 mL of methanol was added, and the solution stirred for 5 min. The organic layer was removed, and the aqueous layer was washed with pentane. The combined organic fractions were filtered through $MgSO_4$, the $MgSO_4$ was washed with additional pentane, and the solvent was carefully removed by rotary evaporation while the flask was cooled in an ice bath. The residual colorless oil was added to a suspension of 3.67 g (4.1 mmol) of *tris*(tetra-*n*-butyl ammonium) hydrogen pyrophosphate in 3 mL of acetonitrile at room temperature. After stirring for 1.5 h, solvent was removed by rotary evaporation to give a thick oil. The residue was dissolved in 3.0 mL of 0.1 M NH_4HCO_3 and applied to a Dowex AG-2 × 8 cation exchange column (2 × 15 cm, NH_4^+ form). The column was eluted with a solution of 0.25 M NH_4HCO_3 and 2 % isopropanol. The eluent was lyophilized to dryness and chromatographed on cellulose [2:1 (v/v) acetonitrile: 0.1 M NH_4HCO_3]. Phosphate-containing fractions were visualized with sulfosalicylic acid/$FeCl_3$. Those containing DMAPP were pooled and lyophilized to give 0.417 g (48 %) of a fluffy white solid; ^{1}H NMR (300 MHz) δ 1.71 (3H, ddd, $^1J_{CH}$ = 125 Hz, $^3J_{CH}$ = 4.5 Hz, $^3J_{CH}$ = 4.5 Hz), 1.75 (3H, ddd, $^1J_{CH}$ = 126 Hz, $^3J_{CH}$ = 4.5 Hz, $^3J_{CH}$ = 4.5 Hz), 4.45 (2H, ddd, $^3J_{HH}$ = 6.9 Hz, $^3J_{PH}$ = 6.9 Hz, $^2J_{CH}$ = 4.1 Hz), 5.44 (2H, dtdd, $^1J_{CH}$ = 151 Hz, $^3J_{HH}$ = 6.9 Hz, $^3J_{CH}$ = 4.5 Hz, $^3J_{CH}$ = 4.5 Hz).

Ethyl [2,4,5-$^{13}C_3$]3-methyl-3-butenoate (9). To a solution of diisopropylamine 0.436 g (4.3 mmol) in 10 mL of THF was added 1.8 mL (4.0 mmol, 2.2 M in hexane) of *n*-butyl lithium at –78 °C. After stirring for 30 min 0.372 g (2.8 mmol) of **7** was added. Stirring was continued for 1 h before rapid addition of 0.95 g of acetic acid in 5 mL of THF. The solution was allowed to warm to room temperature, diluted with 5 mL of ether, and washed with saturated $NaHCO_3$, water and brine. The ethereal solution was then dried over 3 Å sieves and concentrated by distillation to yield a light yellow oil. Analysis by ^{1}H NMR indicated that the oil was a mixture of **7** and **9** in a 15:85 ratio; ^{1}H NMR (300 MHz) showed all of the resonances for **7** and; δ 1.27 (3H, dt, J_{HH} = 7.2 Hz), 1.81 (3H, d, $^1J_{HH}$ = 126 Hz), 2.99 (2H, d, $^1J_{CH}$ = 128 Hz), 4.14 (2H, q, J_{HH} = 7.2 Hz), 4.82 (1H, d, $^1J_{CH}$ = 162 Hz), 4.92 (1H, d, $^1J_{HH}$ = 162 Hz); ^{13}C NMR (125 MHz) δ 14.3, 22.4 (dd, $^3J_{CC}$ = 3.8 Hz, $^3J_{CC}$ = 3.0 Hz), 43.6 (dd, $^3J_{CC}$ = 3.8 Hz, $^3J_{CC}$ = 3.0 Hz), 60.6, 114.5 (dd, $^3J_{CC}$ = 3.0 Hz, $^3J_{CC}$ = 3.0 Hz), 138.68, 171.4 (d, $^1J_{CC}$ = 57.1 Hz); HRMS (EI) $^{12}C_4{}^{13}C_3H_{12}O_2$ calculated 131.0939, found 131.0943.

[2,4,5-$^{13}C_3$]3-Methyl-3-buten-1-ol (10). To a suspension of 0.107 g (2.8 mmol) of lithium aluminum hydride in 2 mL of ether was added 0.185 g (1.4 mmol) of the mixture of **7** and **9**. After stirring for 1 h at room temperature, 110 μL of water was added. Stirring was continued for 10 min before addition of 110 μL of 15 % NaOH. After 10 min, 330 μL of water was added, and the resulting suspension was filtered through a bed of celite. The celite was washed with 10 mL of CH_2Cl_2, and the filtrates were combined. Solvent was removed by rotary evaporation to afford 0.172 g (95 %) of a colorless oil. Analysis by ^{1}H NMR showed that the oil contained alcohols **8** and **10** in a 15:85 ratio; ^{1}H NMR (300 MHz) showed all of the resonances for **8** and: δ 1.76 (3H, ddd, $^1J_{CH}$ = 126 Hz, $^3J_{CH}$ = 4.5 Hz, $^3J_{CH}$ = 4.5 Hz), 2.20 (2H, dtdd, $^1J_{CH}$ = 126 Hz, J_{HH} = 6.3 Hz, $^3J_{CH}$ = 4.5 Hz, $^3J_{CH}$ = 4.5 Hz), 3.72 (2H, td, J_{HH} = 6.3 Hz, $^2J_{CH}$ = 2.1 Hz), 4.79 (1H, ddd, $^1J_{CH}$ = 155 Hz, $^3J_{CH}$ = 4.5 Hz, $^3J_{CH}$ = 4.5 Hz), 4.87 (1H, ddd, $^1J_{CH}$ = 155 Hz, $^3J_{CH}$ = 4.5 Hz, $^3J_{CH}$ = 4.5 Hz); ^{13}C NMR (75 MHz) 22.2 (dd, $^3J_{CC}$ = 3.0 Hz, $^3J_{CC}$ = 3.0 Hz), 40.1 (d,$^1J_{CC}$ = 57.1 Hz), 112.4 (dd, $^3J_{CC}$ = 3.0 Hz, $^3J_{CC}$ = 3.0 Hz), 142.4; HRMS (EI) $^{12}C_2{}^{13}C_3H_{10}O$ calculated 89.0821, found 89.0826.

[2,4,5-$^{13}C_3$]3-Methyl-3-butenyl diphosphate (1). To a solution of *p*-toluenesulfonyl chloride (0.231 g, 1.2 mmol) and 4-(*N,N*-dimethylamino)pyridine (0.201 g, 1.6 mmol) in 1 mL of dry CH_2Cl_2, was added 0.098 g (1.1 mmol) of the mixture of **8** and **10**. The solution was stirred for 5 h. Ether (5 mL) was added, and the mixture was applied to a silica gel column and eluted with ether. Solvent was removed by rotary evaporation to afford 0.149 g (66 %) of a colorless oil which was added to a stirred suspension of *tris*-(tetra-*n*-butylammonium) hydrogen pyrophosphate (1.55 g, 1.72 mmol) in 1.5 mL of acetonitrile at room temperature. The solution was stirred for 3 h before 3 mL of 0.010 M ammonium bicarbonate was added. Acetonitrile was removed by rotary evaporation, the residue was applied to a Dowex AG2-×8 column (2.5 × 10.5 cm, NH_4^+ form), and the column was eluted with 50 mL of 0.01 M NH_4HCO_3. After lyophilization the white solid was purified by preparative HPLC (C-18 Vydac column, elution with 0.10 M NH_4HCO_3). Fractions containing IPP were pooled and lyophilized to afford 0.130 g (71 %) of a fluffy white solid; ^{1}H NMR (300 MHz) δ 1.76 (3H, ddd, $^1J_{CH}$ = 126 Hz, $^3J_{CH}$ = 4.5 Hz, $^3J_{CH}$ = 4.5 Hz), 2.38 (2H, dtdd, $^1J_{CH}$ = 126 Hz, J_{HH} = 6.3 Hz, $^3J_{CH}$ = 4.5 Hz, $^3J_{CH}$ = 4.5 Hz), 4.05 (2H, td, $^3J_{HH}$ = 6.3 Hz, $^2J_{CH}$ = 2.1 Hz), 4.86 (1H, ddd, $^1J_{CH}$ = 155 Hz, $^3J_{CH}$ = 4.5 Hz, $^3J_{CH}$ = 4.5 Hz), 4.87 (1H, ddd, $^1J_{CH}$ = 155 Hz, $^3J_{CH}$ = 4.5 Hz, $^3J_{CH}$ = 4.5 Hz); ^{13}C NMR (75 MHz) 24.5 (dd, $^3J_{CC}$ = 3.0 Hz, $^3J_{CC}$ = 3.0 Hz), 40.7 (ddd, $^3J_{PC}$ = 7.2 Hz, $^3J_{CC}$ = 3.0 Hz, $^3J_{CC}$ = 3.0 Hz), 67.1 (dd, $^3J_{CC}$ = 39.7 Hz, $^2J_{PC}$ = 4.0 Hz, 114.3 (dd, $^3J_{CC}$ = 3.0 Hz, $^3J_{CC}$ = 3.0 Hz), 147.4, ^{31}P {^{1}H} NMR (121 MHz) δ –7.23 (d, $^2J_{PP}$ = 20.2 Hz), –9.41 (d, $^2J_{PP}$ = 20.2 Hz).

Enzymatic reactions

IPP:DMAPP isomerase. Cell paste (2 g) from *E. coli* strain JM101/pIPS241[21] were suspended in 5 mL of extraction buffer (10 mM potassium phosphate, pH 7.0, 10 mM BME, 1 mM PMSF) in a 10 mL centrifuge tube. The cells were disrupted by sonication while being cooled in an ice bath. The homogenate was clarified by centrifugation at 10,000 × *g* for 20 min, diluted to a total volume of 100 mL with extraction buffer, and loaded on to a DE-52 cellulose column (2.5 cm × 20 cm) at a flow rate of 4.0 mL/min. The column was eluted at the same flow rate with extraction buffer until the UV absorbance had returned to

baseline. The phosphate concentration was then increased to 108 mM, and the column was eluted until the absorbance returned to the baseline. IPP isomerase was then eluted as a single peak with 500 mM phosphate, pH 7.0, 10 mM BME. Fractions containing the enzyme were pooled and concentrated to a volume of 2 mL in a Centriprep-10™ concentrator. The solution was stored at 4 °C until used.

Farnesyl diphosphate synthase. Four 1.0 g samples of wet cell paste of *E. coli* strain JM101/pMJY9-11[22] were suspended in 5 mL of extraction buffer (5 mM potassium phosphate, pH 7.0, 10 mM BME, and 1 mM PMSF) and disrupted by sonication while cooling on ice. The homogenate was clarified by centrifugation at 10,000 × *g* for 20 minutes. The supernatant was diluted to 100 mL and a 50 mL portion was loaded on to a DE-52 cellulose column (2.5 cm × 30 cm) equilibrated with 5 mM potassium phosphate, pH 7.0, and 10 mM BME. The column was eluted with starting buffer until the UV absorbance returned to baseline, and FPP synthase was eluted with a linear gradient of 5 to 55 mM phosphate, pH 7.0, and 10 mM BME. Fractions were collected, and those containing activity were combined, concentrated in a Centriprep-10™ concentrator, and assayed for enzymatic activity.

General incubation conditions. Incubations were in 10 mM potassium phosphate buffer, pH 7.0, containing 10 mM $MgCl_2$, 50 mM KCl and 10 mM BME in 12 × 75 mm glass culture tubes. Enzyme(s) was then added, and the mixture was incubated at 37 °C with rotary shaking at 250 rpm. After 4 h, the milky white suspension was spun for 15 min at 14,000 rpm. The pellet was resuspended in 0.5 mL of 25 mM NH_4HCO_3 buffer and applied to a 1 × 3 cm column of Chelex-100. The column was eluted with 10 mL of 1:1 (v/v) 25 mM NH_4HCO_3/acetonitrile. Acetonitrile was removed by rotary evaporation, and the remaining solution was lyophilized. The residue dissolved in 1 mL of 25 mM NH_4HCO_3 and purified by preparative scale HPLC (C-18 Vydac column) by elution with a linear gradient of 25 mM NH_4HCO_3 to acetonitrile over 40 min.

Synthesis of [2,4,6,8,10,12,13,14,15-$^{13}C_9$]FPP (4). A solution of 31 mg (0.10 mmol) of [2,4,5-$^{13}C_3$]IPP in 1.5 mL of buffer containing 0.14 U of IPP isomerase (12 U/mL) and 6.9 U of FPP synthase (8 U/mL) was incubated at 37 °C for 4 h. The material was purified to yield 13.4 mg (88 %) of labeled FPP.

Synthesis of [2,4,13-$^{13}C_3$]FPP (4). A solution of 10 mg (33 μmol) of [2,4,5-$^{13}C_3$]DMAPP and 30 mg (82 μmol) of GPP in 1.5 mL of buffer containing 10.4 U of FPP synthase (8 U/mL) and 0.28 U of IPP isomerase (12 U/mL) were incubated for 4 h at 37 °C. The material was purified to give 12.8 mg (88 %) of labeled FPP.

Synthesis of [10,12,15-$^{13}C_3$]FPP (4). A solution of 10 mg (33 μmol) of **4** and 30 mg (82 μmol) of GPP in 0.5 mL of buffer containing 0.28 U of FPP synthase (8 U/mL) was incubated for 4 h at 37 °C. The material was purified to yield 13.1 mg (90 %) of labeled FPP.

Acknowledgements

This article is dedicated to Professor Bryan Jones on the occasion of his 60th birthday. We thank Dr P. A. Kroon for a cDNA clone of the gene for avian FPP synthase. This research was supported by NIH grants GM 21328 and GM 25982.

References and Notes

1. Poulter, C. D. *Acc. Chem. Res.* **1990**, *23*, 70.

2. Jones, J. B. *Asymmetric Synthesis*, Vol. 5, pp. 309–344, Morrison, J. D., Ed.; Academic Press; New York, 1985.

3. Look, G. C.; Fotsch, C. H.; Wong, C. H. *Acc. Chem. Res.* **1993**, *26*, 182.

4. Abbreviations used are: BME, 2-mercaptoethanol; DIBAL-H, diisobutyl aluminum hydride; DMAP, dimethylamino pyridine; DMAPP, dimethylallyl diphosphate; FPP, farnesyl diphosphate; IPP, isopentenyl diphosphate; LDA, lithium diisopropyl amide; PMSF, phenylmethylsulfonyl fluoride; THF, tetrahydrofuran.

5. Muehlbacher, M.; Poulter, C. D. *Biochemistry* **1988**, *27*, 7315.

6. Poulter, C. D.; Rilling, H.C. *Acc. Chem. Res.* **1978**, *11*, 307.

7. Poulter, C. D. *Acc. Chem. Res.* **1990**, *23*, 70.

8. Clarke, S. *Ann. Rev. Biochem.* **1992**, *61*, 355.

9. Spurgeon, S. L.; Porter, J.W. *Biosynthesis of Isoprenoid Compounds*, Vol. 2, pp. 122, Porter, J. W.; Spurgeon, S. L., Eds; John Wiley; New York, 1981.

10. Wareing, P. F.; Philips, I. D. S. *Growth and Differentiation in Plants*, Chapters 3–5, 3rd ed., Pergamon: New York, 1981.

11. Chen, A.; Zhang, D.; Poulter, C. D. *J. Biol. Chem.* **1993**, *268*, 21701.

12. Croteau, R. *Biosynthesis of Isoprenoid Compounds*, Vol. 1, pp. 225–282, Porter, J. W.; Spurgeon, S. L. Eds; John Wiley; New York, 1981.

13. Cane, D. E. *Biosynthesis of Isoprenoid Compounds*, Vol. 1, pp. 283–374, Porter, J. W.; Spurgeon, S. L., Eds; John Wiley; New York, 1981.

14. Hemming, F. W. *Biosynthesis of Isoprenoid Compounds*, Vol. 2, pp 305–354, Porter, J. W.; Spurgeon, S. L., Eds; John Wiley; New York, 1981.

15. Cornforth, J. W. *Angew. Chemie, Int. Ed.* **1968**, *7*, 903.

16. Clifford, K.; Cornforth, J. W.; Mallaby, R.; Phillips, G. T. *Chem. Commun.* **1971**, 1599.

17. McInnes, A. G.; Wright, J. L. C. *Acc. Chem. Res.* **1975**, *8*, 313.

18. Anderson, M. S.; Muehlbacher, M.; Street, I. P., Proffitt, J.; Poulter, C. D. *J. Biol. Chem.* **1989**, *264*, 19169.

19. Anderson, M. S.; Yarger, J. G.; Burck, C. L.; Poulter, C. D. *J. Biol. Chem.* **1989**, *264*, 19176.

20. Davisson, V. J.; Woodside, A. B.; Stremler, K. E.; Neal, T. R.; Muehlbacher, M.; Poulter, C. D. *J. Org. Chem.* **1986**, *51*, 4768.

21. Street, I. P.; Poulter, C. D. *Biochemistry* **1990**, *29*, 7531.

22. Yan, M. J.; Poulter, C. D. 1994, unpublished results.

23. Arbuzov, A. E.; Razumov, V. *J. Russ. Phys. Chem. Soc.* **1929**, *61*, 623.

24. Wadsworth, W. S.; Emmons, W. D. *J. Am. Chem. Soc.* **1961**, *83*, 1733.

25. Overman, L. E.; Thompson, A. S. *J. Am. Chem. Soc.* **1988**, *110*, 2248.

26. Zhang, D. L.; Poulter, C. D. *Anal. Biochem* **1993**, *213*, 356.

27. Davisson, V. J.; Neal, T. R.; Poulter, C. D. *J. Am. Chem. Soc.* **1993**, *115*, 1235.

28. Davisson, V. J.; Poulter, C. D. *J. Am. Chem. Soc.* **1993**, *115*, 1245.

29. Blanchard, L.; Karst, F. *Gene* **1993**, *125*, 185.

(*Received* 22 *April* 1994)