Supporting Information for

The Mechanism of Dimethylallyltryptophan Synthase: Evidence for A Dimethylallyl Cation Intermediate in an Aromatic Prenyltransferase Reaction

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1. General Information

All reagents were purchased from Sigma-Aldrich and used without further purification unless otherwise noted. D_2O (99.9%) and $H_2{}^{18}O$ (97%) were purchased from Cambridge Isotope Laboratories, Inc. Protein concentrations were determined by the method of Bradford using bovine serum albumin as standard.[1] The steady-state kinetics assays were performed on a Cary 4000 UV-Vis spectrometer with a Cary Temperature Controller attached. Proton-decoupled ^{31}P NMR spectra were recorded on a Bruker AV300 and AV400 spectrometer at 121.5 or 162 MHz, respectively. HPLC analysis was

carried out on a Waters Delta 600 equipped with a model Waters 2996 photodiode array detector using a reverse phase Delta Pak 15μ C18 100Å Waters HPLC column at room temperature (~24 ^{o}C).

2. Purification of Dimethylallyltryptophan Synthase (FgaPT2)

The *fgaPT2* gene of *Aspergillus fumigatus* (strain AF 293) (GenBank accession no. AY775787) flanked with BamH1 and HindIII restriction sites was synthesized and cloned into a pET28a vector (Novagen) by GenScript. Overexpression of FgaPT2 was modified from the protocol described by Steffan *et. al.*[2] Rosetta(DE3) pLysS (Novagen) harboring the recombinant *fgaPT2*/pET28a construct were grown at 37 ^{o}C in 1 L of Terrific Broth (TB) medium containing 35 μg/mL chloramphenicol and 30 μg/mL kanamycin until an OD_{600} of 0.6 was reached. Cells were induced for overexpression by the addition of 119 mg (0.5 mM) of isopropyl-1-thio-*β*-D-galactopyranoside (IPTG). After growing at 24 ^{o}C for an additional 24 h, cells were harvested and FgaPT2 synthase was purified following a previously reported protocol.[2] Typically ~35 mg of enzyme was purified from 1 L culture, and it could be stored at -80 ^{o}C for at least 6 months without significant loss in activity. The SDS-PAGE analysis of purified dimethylallyltryptophan synthase (FgaPT2) is shown in Figure S1.

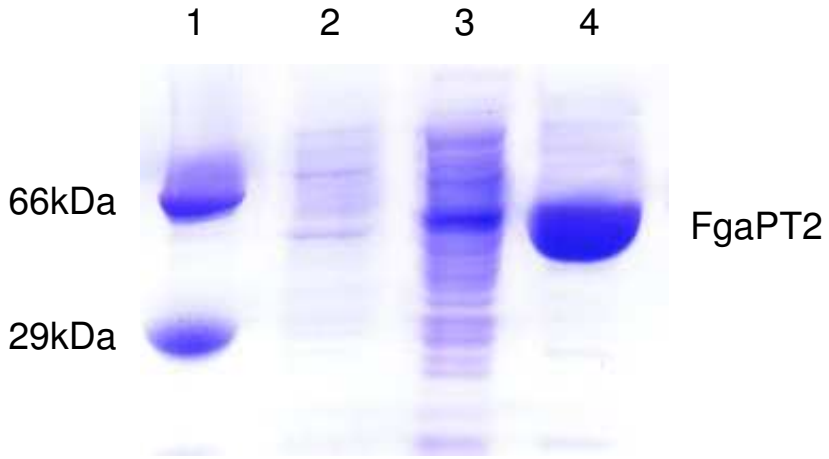


Figure S1. SDS-PAGE gel showing the purification of dimethylallyltryptophan synthase (DMATS). Lane 1 contains molecular mass standards of 66 kDa (bovine serum albumin) and 29 kDa (carbonic anhydrase); lane 2 shows crude cell extract before isopropyl-β-D-1-thiogalactopyranoside (IPTG) induction; lane 3 shows crude cell extract after induction; lane 4 shows the purified dimethylallyltryptophan synthase

3. Unlabeled and Isotopically labeled [1,1-^{2}H]- and [1-^{18}O]-DMAPP

DMAPP and its isotopologues were synthesized by coupling the corresponding alcohols with *bis*-triethylammonium phosphate in the presence of trichloroacetonitrile as previously described by Thulasiram *et. al.*[3] Syntheses of the isotopically labeled alcohols will be elaborated below.

3.1 [1,1-^{2}H]-3-methyl-2-buten-1-ol

[1,1-^{2}H]-3-methyl-2-buten-1-ol was prepared as previously described.[4] The extent of ^{2}H incorporation was determined to be ≥97% by ^{1}H NMR spectroscopy and ESI-MS.

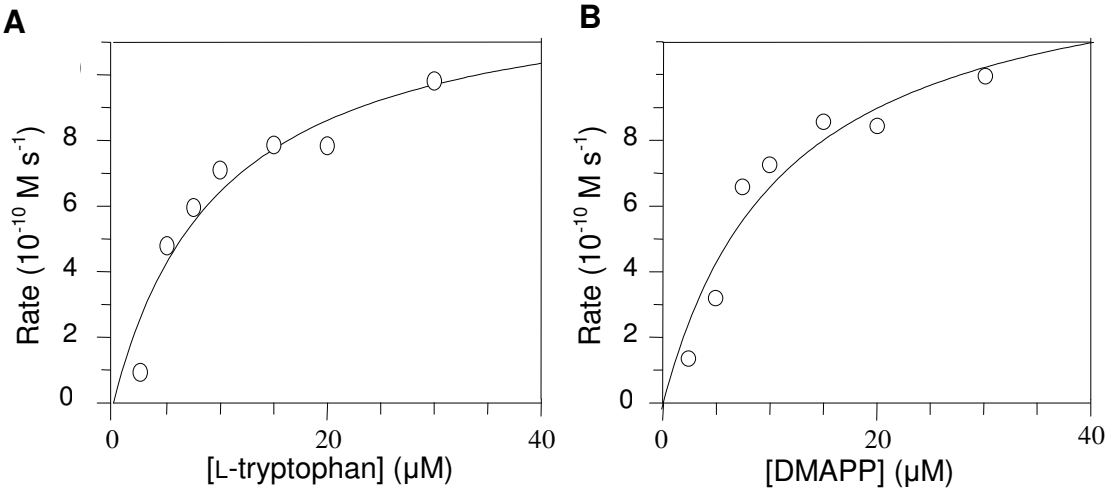
3.2 [1-^{18}O]-3-methyl-2-buten-1-ol

The synthesis of [1-^{18}O]-3-methyl-2-buten-1-ol was modified from the procedure described by Vani *et. al.*[5] 1-Chloro-3-methyl-2-butene (4.27 g, 40.8 mmol) was heated at 100 ^{o}C for 4 hours in the presence of triethylbenzylammonium chloride (TEBA) (0.104g, 0.45 mmol) and sodium [1-$^{18}O_2$]-acetate (3.87 g, 47.1 mmol), which was prepared as previously described.[6] The resultant acetylated product was then extracted with dichloromethane (3 x 10 mL) and concentrated to dryness under vacuum. The residue was dissolved in 30 mL of anhydrous methanol solution containing sodium methoxide (1.3 M) and refluxed for 4 h. The reaction was quenched by addition 20 mL saturated NH_4Cl solution and extracted three times with 20 mL diethyl ether. The organic layer was separated, dried with $MgSO_4$ and evaporated to dryness under reduced pressure. The product [1-^{18}O]-3-methyl-2-buten-1-ol was purified by Kugelrohr distillation. The extent of ^{18}O incorporation was determined to be ~92% by ESI-MS.

4. Continuous Coupled Enzymatic Assay

Enzyme kinetics were measured by a modification of a previously reported continuous coupled phosphate assay.[7] A cuvette containing 50 mM Tris-HCl buffer (pH 7.5, final volume 1000 μL), L-tryptophan (variable), dimethylallyl diphosphate (variable), 2-amino-6-mercapto-7-methylpurine ribonucleoside (MESG) (200 μM), purine nucleoside phosphorylase (PNPase) (1 units exchanged twice into 50 mM Tris-HCl buffer pH 7.5), inorganic pyrophosphatase (PPase) (0.5 units), $MgCl_2$ (5 mM), $CaCl_2$ (5 mM) was thermally equilibrated for 5 min at 35 ^{o}C. The enzymatic reaction was initiated by the addition of DMAT synthase (1 μg) and the rate was calculated from the observed

increase of absorption at 360 nm (using ε = 11,000 $M^{-1} \cdot cm^{-1}$). The K_M value for L-tryptophan was measured in the presence of 200 μM DMAPP (saturating), and that for DMAPP was measured in the presence of 200 μM L-tryptophan (saturating). Kinetic parameters were determined from the initial velocities fit to Michaelis-Menten kinetics using the program *Grafit*, and the hyperbolic curves are shown in Figure S2.



varying [substrate]	K_M (μM)	k_{cat} (s^{-1})
DMAPP	11 ± 4	0.27 ± 0.05
L-tryptophan	10 ± 4	

Figure S2. Enzyme kinetics for dimethylallyltryptophan synthase with natural substrate. (**A**) L-tryptophan as the variable substrate with saturating DMAPP (200 μM); (**B**) DMAPP as the variable substrate with saturating L-tryptophan (200 μM).

4.1 Metal Dependence Experiment

In the metal dependence experiment, two cuvettes containing MESG (200 μM), PNPase (1 unit exchanged twice into 50 mM Tris-HCl buffer, pH 7.5), inorganic PPase (0.5 units) and $MgCl_2$ (5 mM or 8 mM) in Tris-HCl buffer (50 mM, pH 7.5, total volume 900 μL) were thermally equilibrated for 1 min at 35 ^oC and set as background at A_{360}. In separate vials, solutions containing saturating L-tryptophan (200 μM), saturating DMAPP

(200 μM) and either EDTA (3 mM) or $CaCl_2$ (5 mM) in Tris-HCl buffer (50 mM, pH 7.5, total volume 100 μL) were prepared. To these vials was added DMAT synthase (1 μg). After incubating at 35 ^{o}C for 30 s, the contents of the vials were added to the cuvettes (EDTA sample to the 8 mM $MgCl_2$ cuvette and $CaCl_2$ sample to the 5 mM $MgCl_2$ cuvette). The pyrophosphate concentration was measured as a burst of absorbance at 360 nm.

5. Kinetic Isotope Effect Studies

An intermolecular competitive method was employed to measure a KIE on the second-order rate constant k_{cat}/K_M, symbolized as $^{D}(V/K)$. A mixture of two isotopologues of known isotopic composition is treated with the enzyme. After a certain fractional conversion to products has occurred, the isotopic composition of the starting material is recovered and determined. If there is a KIE, the faster reacting isotopologue will be depleted to a greater extent than the slower reacting isotopologue. By accurate measurement of the reaction extent and the initial and final isotopic ratios of the substrates, $^{D}(V/K)$ can be extrapolated using the equation (**1**) derived by Melander and Saunders:[9]

$$^{D}(V/K) = \frac{\ln(1-F_H)}{\ln[(1-F_H)R/R_o]} \quad (\mathbf{1})$$

where F_H is the fractional conversion of the protiated species to products, and R_0 and R are the initial and final ratios of the protiated to deuterated substrate, respectively. The final and initial ratios of deuterated substrate, a_D, to protiated substrate molecules, a_H can be described in Equation 2:

$$R = \frac{a_D}{a_H} \quad (2)$$

5.1 Kinetic Isotope Effect Measurement for [1,1-^{2}H]-Dimethylallyl Diphosphate

To determine the $^D(V/K)$ of dimethylallyl diphosphate, a solution of an approximately 1:1 molar ratio of unlabeled and [1,1-^{2}H]-dimethylallyl diphosphate (12 mM each), 26 mM L-tryptophan and 5 mM $CaCl_2$ in Tris-HCl (pH 7.5, 50 mM, final volume 12 mL) was prepared and DMAT synthase (1.2 mg) was added. Two aliquots (1 mL each) were collected at 0, 90, 120 and 240 min time intervals for ESI-MS and NMR analyses. The ESI-MS samples were quenched by adding 1 mL of dry-ice-chilled methanol and the enzyme was removed by ultrafiltration (Amicon Ultra-4, 10 000 MWCO) prior to negative ESI-MS data collection. The NMR samples were immediately diluted with 10 mL of distilled water, flash frozen with liquid N_2 and lyophilized. ^{31}P NMR spectra (D_2O containing 10 mM 2-deoxy-glucose-6-phosphate) were collected with an AV300 NMR spectrometer at 4.5 s delay time. F_H was calculated by integration the α phosphate signal at -10.0 ppm to the internal standard 2-deoxy-glucose-6-phosphate (10 mM) at 4.0 ppm in the ^{31}P NMR spectra before and after enzyme additions. R_o and R were calculated from the mass spectra before and after addition of the enzyme using the relative peak intensities of the protiated substrate [DMAPP *m/z* 245 (M – H^-)] to the deuterated substrates [[1,1-^{2}H]-DMAPP *m/z* 247 (M – H^-)] (Figure S3). The measurements were performed in triplet and an average value was reported. The values of individual trials gave the following results: $^D(V/K)$ = 1.13, 1.16, and 1.18.

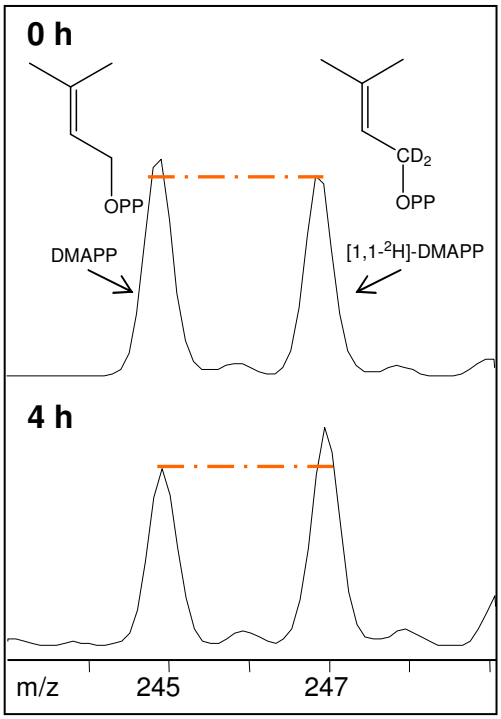


Figure S3. Mass spectra following the consumption of a mixture of unlabeled DMAPP and [1,1-^{2}H]-DMAPP in the reaction catalyzed by dimethylallyltryptophan synthase. Top spectrum was taken prior to the addition of the enzyme, and bottom spectrum after 74% of the unlabeled DMAPP has been converted to product.

5.2 Kinetic Isotope Effect Measurement for [4-^{2}H]-L-Tryptophan

[4-^{2}H]-L-tryptophan was synthesized by photosubstitution as previously described.[10] The extent of ^{2}H incorporation at C-4 was determined to be 82% by ^{1}H NMR spectroscopy and ESI-MS. Minor deuteration was also observed at the C-2 and C-7 positions during the reaction, and caused 16% of the labeled product to be di-deuterated.

To determine the $^D(V/K)$ of L-tryptophan, a solution of an approximately 1:1 molar ratio of [4-^{1}H] and [4-^{2}H]-L-tryptophan (12 mM each), 26 mM dimethylallyl diphosphate and 5 mM $CaCl_2$ in Tris-HCl (pH 7.50, 50 mM, final volume 12 mL) was prepared and 1.2 mg of DMAT synthase was added. 1 mL aliquots were collected at 0, 90, 120 and 240 min time intervals. Each sample was quenched by the addition of 1 mL of

dry-ice-chilled methanol containing 10 mM L-1-methyl-tryptophan and the protein was removed by ultrafiltration (Amicon Ultra-4, 10 000 MWCO). The samples were subjected to HPLC analysis under the previously described conditions,[11] and the L-tryptophan peaks were collected for positive ESI-MS analysis. F_H values were calculated from the HPLC data before and after addition of the enzyme by integration of the L-tryptophan peak relative to the internal standard peak of L-1-methyl-tryptophan. R_o and R were calculated from the intensity of the mass spectral signals before and after addition of the enzyme (L-tryptophan *m/z* 205 ($M + H^+$) and [4-^{2}H]-L-tryptophan *m/z* 206 ($M + H^+$)) (Figure S4). The intensities of the signals were corrected for the natural abundance of ^{13}C and for the presence of the di-deuterated tryptophan (m/z 207 $M + H^+$) before R_o and R were calculated. The measurements were performed in triplicate and an average value was reported. The values of individual trials were gave the following results $^D(V/K)$ = 0.78, 0.82, and 0.83.

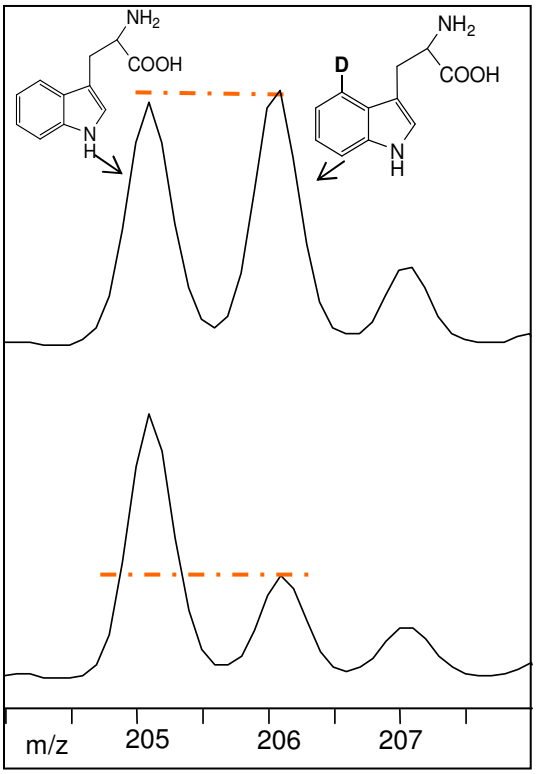


Figure S4. Mass spectra following the consumption of a mixture of unlabeled L-tryptophan and [4-^{2}H]-L-tryptophan in the reaction catalyzed by dimethylallyltryptophan synthase. The top spectrum was taken prior to the addition of the enzyme, and the bottom spectrum after 70% of the unlabeled L-tryptophan had been converted to product.

6. Positional Isotope Exchange (PIX) experiment

A 1 mL solution containing [1-^{18}O]-DMAPP and unlabeled DMAPP (30 mM total concentration, 63% ^{18}O incorporation) and L-tryptophan (24 mM, 0.8 equivalent) in Tris-HCl prepared using D_2O (50 mM, pD 7.9, containing 3 mM EDTA) was placed in a 5 mm NMR tube and a ^{31}P NMR spectrum was collected. A solution of DMAT synthase (1 mg in 1 mL Tris-HCl 50 mM pH 8) was added and incubated for 4 h at 35 ^{o}C. The enzyme was removed by ultrafiltration (Amicon Ultra-4, 10 000 MWCO) and to the filtrate was added Chelex-100 resin (20 mg of 200-400 mesh, Na^{+} form, previously rinsed with D_2O). After extensive mixing, a second ^{31}P NMR spectrum was

obtained. The proton-decoupled ^{31}P NMR spectra were obtained on a Bruker AV300 multinuclear spectrometer operating at a frequency 121.5 MHz. All spectra were externally referenced to H_3PO_4 (0 ppm). Typical acquisition parameters were 2437 Hz (20 ppm) sweep width centered at -5 ppm, 27 s acquisition time, 2 s delay time and 13.5 μs pulse width. Well resolved spectra were achieved after 200 to 1000 scans. The FIDs were apodized using an Exponential/Gaussian resolution enhancement function. The ^{31}P NMR spectrum showing both the α and β phosphorus atoms of [1-^{18}O]-dimethylallyl diphosphate in the PIX reactions is depicted in Figure S5, L-Trp rxn.

6.1 Estimation of Partitioning Ratio

In the DMAT synthase-catalyzed PIX reaction the isotopic scrambling reflects a partitioning of the dimethylallyl cation/pyrophosphate ion pair between an irreversible conversion to products and a reversible return to the DMAPP starting material. Since no scrambling is observed in the absence of tryptophan, the initial rate of PIX will correlate linearly with the rate of product formation at all substrate concentrations. Therefore, at low levels of observed PIX, the partitioning ratio can be calculated from the ratio of the percentage of scrambled DMAPP to the percentage of DMAPP that has been converted to product. In the PIX experiment with [1-^{18}O]-DMAPP and tryptophan, the amount of scrambled DMAPP does not exceed 15% over the course of the first 60% of the reaction (effectively initial velocity conditions with respect to scrambling), and an approximate value of the partitioning ratio (v_{ex}/v_{chem}) can be obtained from equation (3):

v_{ex}/v_{chem} = percentage scrambled DMAPP/percentage DMAPP converted to product (**3**)

The percentage of scrambled DMAPP was calculated by dividing the integral of the ^{31}P NMR signal due to the α-phosphorus of DMAPP bearing a non-bridging ^{18}O label (Figure 2b, -9.31 ppm) by the combined integrals of the signals due to the α-phosphorus of DMAPP bearing either a bridging or non-bridging ^{18}O label (Figure 2b, -9.30 and -9.31 ppm). This value was found to be 15%.

The percentage of DMAPP converted to product was calculated by dividing the integration of the pyrophosphate product signals by the combined integrations of the pyrophosphate signals and the total signals due to the DMAPP α-phosphorus (both labeled and unlabeled) X 2. This value was found to be 57%. Therefore, v_{ex}/v_{chem} = 15/57 = 0.26.

6.2 PIX experiment with fluorinated L-tryptophan analogs

PIX experiments using 6-fluoro-D,L-tryptophan (6F-D,L-Trp) were performed as described above, but with an excess of 6F-D,L-Trp (70 mM, 2.3 equivalents relative to DMAPP) and 2 mg DMAT synthase. Proton-decoupled ^{31}P NMR spectroscopy was conducted as described above except that the delay time between each scan was increased to 3 s. The ^{31}P NMR spectrum showing both the α and β phosphorus atoms of [1-^{18}O]-dimethylallyl diphosphate in the PIX reaction is depicted in Figure S5, 6-F-Trp rxn.

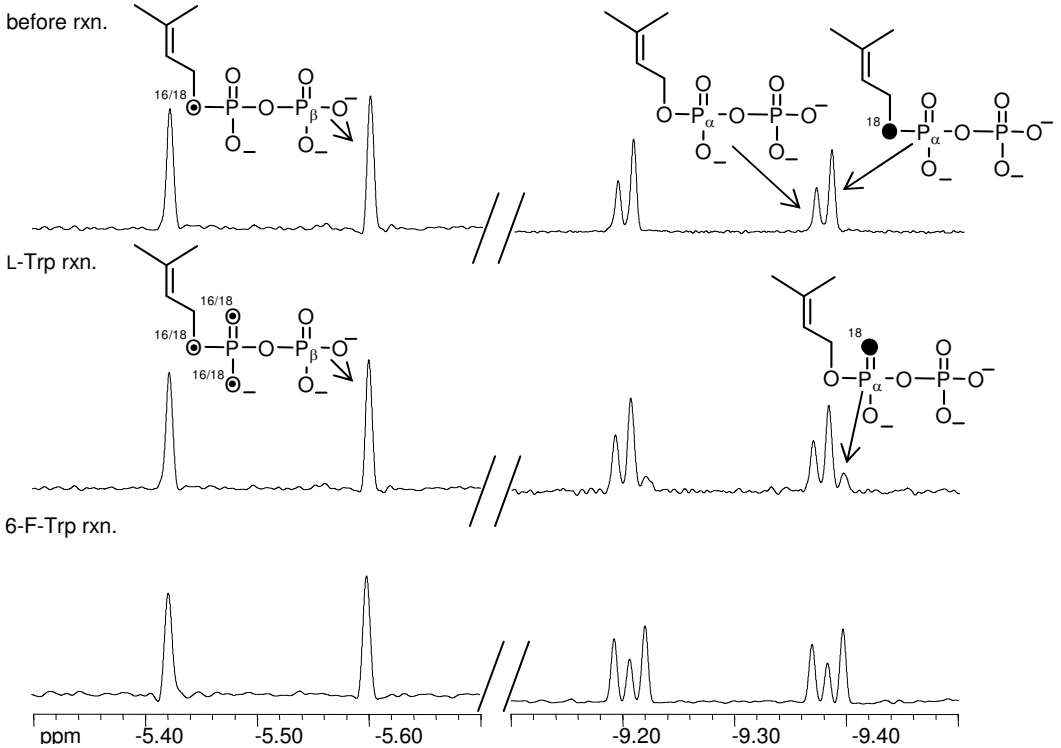


Figure S5. ^{31}P NMR analysis of [1-^{18}O]-dimethylallyl diphosphate in the reaction catalyzed by dimethylallyltryptophan synthase. Spectra on the left and right show the doublets of the β- and α-phosphorous atoms of the ^{18}O-labeled DMAPP, respectively.

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