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Syntheses of deuterium labeled prenyldiphosphate and prenylcysteine analogues for *in vivo* mass spectrometric quantification

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A Wittig reaction employing $Li(CD_3)_2CP(C_6H_5)_3$ was used to prepare d_6-farnesol and d_6-geranylgeraniol. Reductive amination of aniline-2,3,4,5,6-d_5 was used to prepare the unnatural isoprenoid analogues d_5-anilinogeraniol and d_5-anilinofarnesol. All of these deuterated isoprenols were elaborated into their diphosphate and cysteine thioether derivatives suitable for use as stable-isotope labeled standards for quantitative mass spectrometric analysis.

Keywords: FPP; AGPP; GGPP; AFPP; FTase; protein prenylation; mass standards; stable labeled synthesis

Introduction

The mevalonate isoprenoid biosynthesis pathway provides the cell with critical metabolic intermediates that are involved in multiple cellular processes.[1,2] Inhibition of enzymes in this pathway with statins and bisphosphonates is clinically used in the respective treatment of hypercholesterolemia and metabolic bone disease.[3–21] Farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP) are two important isoprenoid intermediates at branch points of this pathway. Both FPP and GGPP serve as prenyl donors for protein isoprenylation, which is essential for many biological functions such as cell growth and cell proliferation. Because protein isoprenylation is an important therapeutic target in cancer research, there is substantial interest in understanding the role that isoprenoid levels play in disease pathology.[22] Cells can take up both natural and unnatural isoprenols from the exogenous media and appear to convert them to the corresponding diphosphates for use in protein isoprenylation.[23–30] In addition, natural and unnatural isoprenoid diphosphates are converted to their corresponding isoprenols by the action of specific phosphatases.[31] The unnatural FPP and GGPP analogues anilinogeranyl diphosphate (AGPP) and anilinofarnesyl diphosphate (AFPP) are particularly useful for studying isoprenoid metabolism and protein isoprenylation because of the desirable biochemical and mass spectrometric properties of the aniline moiety.[31–35] The accurate, quantitative and easy measurement of isoprenoid diphosphate levels and the products of protein isoprenylation are critical to increase our knowledge about the regulation of isoprenoid metabolism. Liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) methods for detecting isoprenoid diphosphates are two orders of magnitude more sensitive than analysis by radio-HPLC.[36] Accurate and sensitive quantitative LC–MS/MS analysis is greatly facilitated by employing stable-isotope labeled standards.[37]

We report the synthesis of series of natural and unnatural, stable, deuterium labeled isoprenoid analogues suitable as standards for quantitative mass spectrometric analysis and for metabolic labeling studies. Previous preparations of d_6-farnesol introduced the isotope in multiple steps.[38,39] In our strategy, common intermediates for the desired d_6-farnesyl and d_6-geranylgeranyl derivatives were prepared by modifying a previously reported synthesis of d_6-geranyldiphosphate to simultaneously incorporate all of the deuteriums via a Wittig reaction.[40] Analogously, the isotope was simultaneously incorporated into d_5-anilinogeranyl and d_5-anilinofarnesyl derivatives by reductive amination of aniline-2,3,4,5,6-d_5.

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Results and discussion

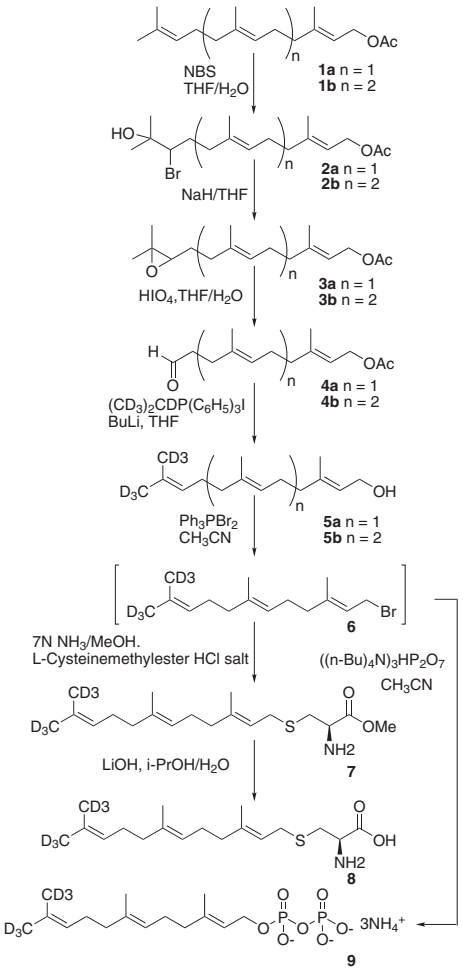
The preparation of hexadeuterated farnesyl and geranylgeranyl derivatives is outlined in Scheme 1. Aldehydes **4a** and **4b** were prepared in three steps from farnesyl acetate or geranylgeranyl acetate respectively using a slight modification of reported procedures.[41–43] Epoxides **3a–b** were obtained by hydrobromination of farnesyl acetate **1a** or geranylgeranyl acetate **1b** followed by ring closure of bromohydrins **2a–b** with NaH in THF. Oxidation of epoxides **3a–b** with hydroiodic acid provided the desired aldehydes **4a–b**. The key intermediates d_6-farnesol **5a** and d_6-geranylgeraniol **5b** were prepared in 39% yield by reaction of aldehydes **4a–b** with 1.0 equivalent of the anion of $(CD_3)_2CDP(C_6H_5)_3I$ using BuLi as the base. The phosphonium salt $(CD_3)_2CDP(C_6H_5)_3I$ was prepared by heating 2-iodopropane-d_7 (98 atom % D) and triphenylphosphine in a sealed tube at 130°C for 6 h followed by recrystallization from EtOH/EtOAc. Reaction of d_6-farnesol **5a** with Ph_3PBr_2 in MeCN furnished d_6-farnesylbromide **6**, which was divided into two portions and used without purification to prepare d_6-farnesylcysteine **8** and d_6-farnesyldiphosphate **9**. Trapping bromide **6** with $((n\text{-}Bu)_4N)_3HP_2O_7$ in MeCN gave d_6-FPP **9**, which was converted to the NH_4^+ form by ion exchange chromatography and then purified by RP-HPLC. Condensation of bromide **6** with L-cysteine methyl ester in 7 N NH_3/MeOH provided methyl ester **7**, which was saponified with LiOH/*i*-PrOH to give the desired d_6-farnesylcsyteine **8** in 92% yield after RP-HPLC.

The unnatural isoprenoid analogues **16a** (d_5-AGPP), **16b** (d_5-AFPP), **15a** (d_5-AG-cysteine) and **15b** (d_5-AF-cysteine) were prepared using modified procedures from Spielmann and co-workers and are outlined in Scheme 2.[32] Oxidation of geranyl acetate and farnesyl acetate with SeO_2 provided aldehydes **10a** and **10b**, respectively.[32,43] Reductive amination of aniline-2,3,4,5,6-d_5 (98 atom % D) with aldehyde **10a** and **10b** gave acetates **11a** and **11b**, which were saponified with K_2CO_3 in MeOH/H_2O to provide alcohols **12a** and **12b**. The desired diphosphates **16a–b** and cysteines **15a–b** were elaborated as described for the farnesyl derivatives above.

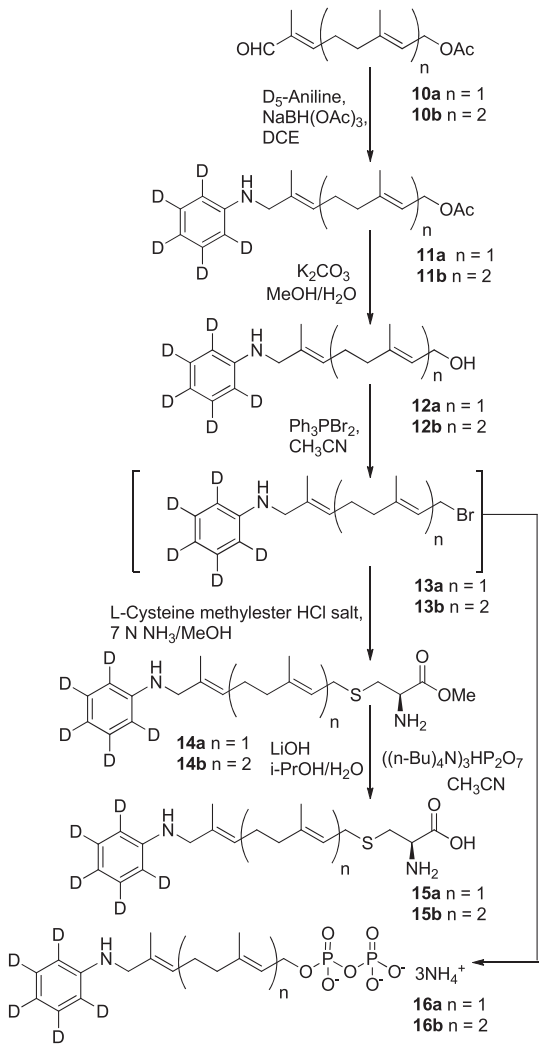
The final diphosphates and cysteines were characterized by ^{1}H-NMR and both low resolution and high resolution MS. In addition, the diphosphates were characterized by ^{31}P-NMR. In all cases, the levels of deuterium isotope incorporation were identical to that in the starting synthons, and there was no evidence of positional scrambling.

Experimental procedure

All RP-HPLC was performed on an Agilent 1200 HPLC system equipped with a microplate autosampler, diode array and fluorescence detector



Scheme 1. Synthesis deuterated isoprenoids



Scheme 2. Synthesis of deuterated unnatural isoprenoids

(Santa Clara, USA). Reaction temperature refers to the external bath. All solvents and reagents were purchased from VWR (Radnor, USA) (EM Science-Omnisolv high purity) and Aldrich (St. Louis, USA), respectively, and used as received. Synthetic products were purified by silica gel flash chromatography (EtOAc/hexane) unless otherwise noted. RP-HPLC purification of lipid diphosphates was carried out using a Varian (Santa Clara, USA) Dynamax, 10 μm, 300 Å, C-18 (10 mm × 250 mm) column and eluted with a gradient mobile phase and flow rate of 4 mL/min: 0–3 min, 90% A; 3–18 min, 0% A; 18–20 min, 0% A; 20–23 min, 90% A; and monitored at 254 and 210 nm [A: Water with 0.1% TFA, B: CH_3CN with 0.1% TFA for cysteine purification; A is 25 mm aqueous ammonium acetate, B is CH_3CN for diphosphates purification]. ^1H NMR and ^{13}C NMR spectra of alcohols were obtained in $CDCl_3$ and ^1H and ^{31}P of diphosphates in D_2O with a Varian Inova spectrometer operating at 400 MHz (^1H), 100.6 MHz (^{13}C) and 161.8 MHz (^{31}P). Chemical shifts are reported in ppm from $CDCl_3$ internal peak at 7.24 ppm for ^1H and 77.4 ppm for ^{13}C (TSP, 0 ppm for 1H; H_3PO_4 as an external reference, 0 ppm for ^{31}P). High resolution electrospray ionization mass spectra were recorded with an AB Sciex Triple TOF 5600 instrument at a resolution greater than 26,000. C17 Lysophosphatidyl choline with a mass of 510.3554 was used as an internal reference to calibrate the spectrum.

Syntheses of (2*E*,6*E*)-10-bromo-11-hydroxy-3,7,11-trimethyl dodeca-2,6-dien-1-yl acetate 2a and (2*E*,6*E*,10*E*)-14-bromo-15-hydroxy-3,7,11,15-tetramethylhexadeca-2,6,10-trien-1-yl acetate 2b

The synthesis of **2a** and **2b** was accomplished using a modification of published procedures.[42,44]

To a stirred solution of farnesyl acetate **1a** (4.68 g, 17.7 mmol) in THF/H_2O (100 mL:50 mL) at 0°C was added *N*-bromosuccinimide (3.53 g, 31.1 mmol) in several portions over 1 h and then stirred for 4 h. EtOAc (200 mL) was added, and the reaction mixture was allowed to warm up to rt. The organic phase was separated, and the aqueous phase was extracted EtOAc (3 × 200 mL), the combined organic layers were washed with brine, dried over $MgSO_4$ and concentrated under reduced pressure. The crude product was purified by silica gel column chromatography to give compound **2a** (4.28 g, 67%). Geranylgeranyl acetate **1b** (170 mg) gave 147.4 mg of **2b** (67%).

2a ^1H NMR ($CDCl_3$) δ 1.31 (s, 3H), 1.33 (s, 3H), 1.57 (s, 3H), 1.68 (s, 3H), 1.70–1.8 (m, 2H), 1.90–2.0 (m, 2H), 2.03 (s, 3H), 2.04–2.15 (m, 2H), 2.24–2.32 (m, 2H), 3.94 (dd, *J* = 1.6,11.2 Hz, 1H), 4.57 (d, *J* = 6.8 Hz, 2H), 5.15–5.18 (m, 1H), 5.29–5.34 (m, 1H).

2b ^1H NMR ($CDCl_3$) δ 1.32 (s, 3H), 1.33 (s, 3H), 1.58 (s, 6H), 1.69 (s, 3H), 1.69–1.82 (m, 2H), 1.92–2.13 (m, 11H), 2.27–2.31 (m, 2H), 3.96 (dd, *J* = 1.6, 11.2 Hz, 1H), 4.57 (d, *J* = 7.2 Hz, 2H), 5.09 (t, *J* = 6.0 Hz, 1H), 5.18 (t, *J* = 6.8 Hz, 1H), 5.33 (t, *J* = 6.8 Hz, 1H).

Syntheses of (2*E*,6*E*)-9-(3,3-dimethyloxiran-2-yl)-3,7-dimethylnona-2,6-dien-1-yl acetate 3a and (2*E*,6*E*,10*E*)-13-(3,3-dimethyloxiran-2-yl)-3,7,11-trimethyltrideca-2,6,10-trien-1-yl acetate 3b

To a stirred solution of bromohydrin **2a** (3.68 g, 10.22 mmol) in THF (70 mL) was added sodium hydride (673 mg, 16.8 mmol) in one portion and then stirred for 2 h. The reaction was quenched with 2 mL saturated ammonium chloride and extracted with EtOAc (50 mL 3). The combined extracts were washed with brine, dried over $MgSO_4$ and concentrated under reduced pressure to give epoxide **3a** (2.6 g, 93%). Bromohydrin **2b** (742 mg) gave 579 mg of epoxide **3b** (99%).

3a ^1H NMR ($CDCl_3$) δ 1.26 (s, 3H), 1.30 (s, 3H), 1.59–1.60 (m, 2H), 1.62 (s, 3H), 1.70 (s, 3H), 2.05 (s, 4H), 2.06–2.22 (m, 6H), 2.69 (t, 6.4 Hz, 1H), 4.58 (d, *J* = 7.2 Hz, 2 Hz), 5.13–5.16 (m, 1H), 5.32–5.36 (m, 1H).

3b ^1H NMR ($CDCl_3$) δ 1.25 (s, 3H), 1.29 (s, 3H), 1.55 (s, 6H), 1.59 (s, 3H), 1.96–2.10 (m, 15H), 2.69 (t, *J* = 7.6 Hz, 1H), 4.58 (d, *J* = 7.2 Hz, 2H), 5.09 (t, *J* = 6.8 Hz, 1H), 5.15 (t, *J* = 6.8 Hz, 1H), 5.34 (t, *J* = 6.8 Hz, 1H).

Syntheses of (2*E*,6*E*)-3,7-dimethyl-10-oxodeca-2,6-dien-1-yl acetate 4a and (2*E*,6*E*,10*E*)-3,7,11-trimethyl-14-oxotetradeca-2,6,10-trien-1-yl acetate 4b

To a solution of epoxide **3a** (2.5 g, 8.93 mmol) in 60 mL 1:1 THF/H_2O was added 2.6 g of periodic acid (11.4 mmol), stirred at rt for 2 h, extracted with EtOAc (3 × 50 mL), dried over $MgSO_4$ and concentrated under reduced pressure. The crude aldehyde was purified by silica gel column chromatography using EtOAc/hexanes with 0.5% triethylamine as solvent to give **4a** (1.9 g, 90%). Epoxide **3a** (100 mg) gave 54 mg of aldehyde **4b** (61%).

4a ^1H NMR ($CDCl_3$) δ 1.61 (s, 3H), 1.69 (s, 3H), 2.05 (s, 3H), 2.07 2.12 (m, 2H), 2.31 (t, *J* = 7.6 Hz, 2H), 2.48–2.52 (m, 2H), 4.57 (d, *J* = 7.2 Hz, 2H), 5.09–5.13 (m, 1H), 5.29–5.34 (m, 1H), 9.74 (t, *J* = 2.0 Hz, 1H).

4b ^1H NMR ($CDCl_3$) δ 1.58 (s, 3H), 1.60 (s, 3H), 1.70 (s, 3H), 1.95–1.98 (m, 2H), 2.04–2.15 (m, 6H), 2.30 (t, *J* = 7.2 Hz, 2H), 2.47–2.52 (m, 2H), 4.58 (d, *J* = 6.8 Hz, 2H), 5.09 (t, *J* = 6.8 Hz, 1H), 5.13 (t, *J* = 6.8 Hz, 1H), 5.33 (t, *J* = 6.8 Hz, 1H), 9.74 (t, *J* = 2.0 Hz, 1H).

Syntheses of (2*E*,6*E*)-3,7-(11-d_3)-trimethyldodeca-(12-d_3)-2,6,10-trien-1-ol 5a and (2*E*,6*E*,10*E*)-3,7,11-(15-d_3)-tetramethylhexadeca-(14-d_3)-2,6,10,14-tetraen-1-ol 5b

The required isopropyl-d_7-triphenylphosphonium iodide was prepared by heating a mixture of isopropyliodide-d_7 (2 g, 11.3 mmol) and triphenylphosphine (3.07 g, 11.72 mmol) in a sealed tube at 130°C for 6 h, followed by recrystallizing the resultant crude product from ethanol (3.13 g, 63%). To the d_7-isopropyl triphenylphosphonium iodide (4.6 g, 10.5 mmol) dissolved in THF(25 mL) was added a 2.5-M solution of BuLi (4.2 mL, 10.5 mmol) dropwise at −20°C and stirred for 1 h before adding aldehyde **4a** (2.51 mg, 10.5 mmol) in THF (10 mL). The reaction mixture was stirred for another 4 h at −20°C and quenched with saturated NH_4Cl solution (5 mL). The resultant mixture was extracted with CH_2Cl_2 (3 × 50 mL), and then the combined organic extracts were washed with brine, dried over $MgSO_4$ and concentrated at reduced pressure. The crude product was purified by silica gel column chromatography to give pure d_6-farnesol (911 mg, 38%). **4a** (74 mg) gave 25 mg of **5b** (35%).

5a ^1H NMR ($CDCl_3$) δ 1.58 (s, 3H), 1.66 (s, 3H), 1.94–2.12 (m, 8H), 4.13 (d, *J* = 7.6 Hz, 2H), 5.05–5.12 (m, 2H), 5.38–5.42 (m, 1H).

^{13}C NMR ($CDCl_3$) δ 15.98, 16.25, 26.23, 26.66, 45.00, 59.38, 123.32, 123.75, 124.33, 131.17, 135.37, 139.84.

HR mass *m/z* ($M-H_2O+1$) 211.2334

5b ^1H NMR ($CDCl_3$) δ 1.59 (s. 3H), 1.60 (s, 3H), 1.68 (s, 3H), 1.95–2.13 (m, 12H), 4.15(d, *J* = 6.4 Hz, 2H), 5.08–5.12 (m, 3H), 5.42 (t, *J* = 7.2 Hz, 1H)

^{13}C NMR ($CDCl_3$) δ 16.02, 16.29, 26.31, 26.61, 26.74, 39.56, 39.68, 39.73, 59.43, 123.28, 123.76, 124.15, 124.40, 131.11, 134.98, 135.40, 139.90

HR mass *m/z* ($M-H_2O+1$) 279.2960

Syntheses of (*R*)-methyl 2-amino-3-(((2*E*,6*E*)-3,7,11-d_3-trimethyldodeca-12-d_3-2,6,10-trien-1-yl)thio)propanoate 7

To a 0°C solution of d_6-farnesol (150 mg, 0.66 mmol) in acetonitrile (10 mL) was added dibromotriphenylphosphorane (285 mg, 0.67 mmol) in CH_3CN (10 mL). The reaction mixture was stirred for 8 h, and then a solution of L-cysteinemethylester HCl salt (2.26 g, 13.0 mmol) in 7 M NH_3/MeOH (40 mL) was added. The resultant reaction mixture was stirred at 0°C overnight, warmed to rt and then extracted with Et_2O (5 × 50 mL). The combined ether extracts were concentrated under reduced pressure and purified by silica gel column chromatography to give compound **7** (96.5 mg, 43% in two steps).

^1H NMR ($CDCl_3$) δ 1.59 (s, 3H), 1.67 (s, 3H), 1.94–2.10 (m, 8H), 2.81 (dd, *J* = 7.6, 14.0 Hz, 1H), 2.98 (dd, *J* = 4.40,13.76 Hz, 1H), 3.14–3.26 (m, 2H), 3.76 (s, 3H), 3.80–3.86 (m, 2H), 5.06–5.12 (m, 2H), 5.22 (t, *J* = 8.0 Hz, 1H)

LR mass *m/z* (M + 1) 346.3

Synthesis of (*R*)-2-amino-3-(((2*E*,6*E*)-3,7,11-d_3-trimethyldodeca-12-d_3-2,6,10-trien-1-yl)thio)propanoic acid 8.

A mixture of compound **7** (68 mg, 0.197 mmol) and LiOH (47 mg, 1.9 mmol) was stirred in 1:1 water/*i*-PrOH (2 mL) overnight at rt. The pH of resultant reaction mixture was brought to 3 by adding drops of 1 N HCl. The cloudy solution was filtered through a syringe filter (Whatman, 13 mm CD/X disposable polypropylene prefilter). The clear solution was purified by RP-HPLC (C_{13} column) using CH_3CN/H_2O (0.1% TFA) to give compound **8** (51.5 mg, 79%).
^{1}H NMR (CD_3OD) δ 1.60 (s, 3H), 1.73 (s, 3H), 1.94–2.14 (m, 8H), 2.87 (dd, *J* = 8.8, 14.8 Hz, 1H), 3.13 (dd, *J* = 3.8, 14.8 Hz, 1H), 3.19–3.26 (m, 1H), 4.03 (dd, *J* = 3.6, 8.8 Hz, 1H), 5.06–5.12 (m, 2H), 5.25 (t, *J* = 8.4 Hz, 1H)
HR mass *m/z* (M + 1) 332.2528

Synthesis of (2*E*,6*E*)-3,7,11-d_3-trimethyldodeca-12-d_3-2,6,10-trien-1-yldiphosphate 9.

To the *in situ* made bromide **6** from alcohol **5** (25 mg, 0.11 mmol) in acetonitrile (5 mL) at 0°C was added ($(n\text{-}Bu)_4N)_3HP_2O_7$ (430 mg, 0.44 mmol) in CH_3CN (10 mL). The resultant reaction mixture was stirred for 3 h at rt and then concentrated. The resultant residue was washed with Et_2O (5 mL), the organic wash discarded, the residue suspended in 25 mm NH_4HCO_3 solution containing 2% *i*-PrOH (4 mL) and purified as for compound **7** (16 mg, 32%).
d_6-FPP ^{1}H NMR (D_2O) δ 1.48 (s, 3H), 1.58 (s, 3H), 1.86–1.90 (m, 2H), 1.95–2.03 (m, 2H), 4.33 (t, *J* = 6.8 Hz, 2H), 5.03–5.09 (m, 2H), 5.33 (t, *J* = 6.8 Hz, 1H)
^{31}P NMR (D_2O) δ −6.95 (d, *J* = 24.2 Hz, 1P), −10.54 (d, *J* = 24.2 Hz, 1P)
HR mass *m/z* 387.1596 (M − H)

Syntheses of (2*E*,6*E*)-3,7-dimethyl-8-oxoocta-2,6-dien-1-yl acetate **10a** and (2*E*,6*E*,10*E*)-3,7,11-trimethyl-12-oxododeca-2,6,10-trien-1-yl acetate **10b** were accomplished by following reported procedures.[32]

Syntheses of (2*E*,6*E*)-3,7-dimethyl-8-(2,3,4,5,6-d_5-phenyl amino)octa-2,6-dien-1-yl acetate 11a and (2*E*,6*E*,10*E*)-3,7,11-trimethyl-12-(d_5-phenylamino)dodeca-2,6,10-trien-1-yl acetate 11b

A mixture containing aldehyde **10a** (1 g, 4.8 mmol), d_5-aniline (550 mg, 5.6 mmol) and AcOH (0.3 mL, 315 mg, 5.3 mmol) in DCE (15 mL) was stirred for 20 min at rt. Then, freshly made $NaBH(OAC)_3$ (2.4 mg, 11.4 mmol) was added in THF (25 mL) and stirred overnight before quenching with 5% $NaHCO_3$. The reaction mixture was extracted with CH_2Cl_2 (100 mL × 2), and the combined organic extracts were washed with brine, dried over $MgSO_4$, filtered and concentrated under reduced pressure. The crude product was purified by silica gel column chromatography to give compound **11a** (980 mg, 70%). Aldehyde **10b** (450 mg) gave 422 mg of compound **11b** (73%).
d_5-AGOAc **11a** ^{1}H NMR ($CDCl_3$) δ 1.64 (s, 3H), 1.67 (s, 3H), 2.01–2.06 (m, 5H), 2.12–2.17 (m, 2H), 3.61 (s, 2H), 4.55 (d, *J* = 7.2 Hz, 2H), 5.32–5.32 (m, 1H), 5.34–5.38 (m, 1H)
d_5-AFOAc **11b** ^{1}H NMR ($CDCl_3$) δ 1.56 (s, 3H), 1.63 (s, 3H), 1.67 (s, 3H), 1.94–1.98 (m, 2H), 2.01 (s, 3H), 2.05–2.10 (m, 2H), 3.60 (s, 2H), 4.55 (d, *J* = 7.2 Hz, 2H), 5.05 (t, *J* = 6.8 Hz, 1H), 5.31 (t, *J* = 6.8 Hz, 1H), 5.37 (t, *J* = 6.8 Hz, 1H)

Syntheses of (2*E*,6*E*)-3,7-dimethyl-8-(2,3,4,5,6-d_5-phenyl amino)octa-2,6-dien-1-ol 12a and (2*E*,6*E*,10*E*)-3,7,11-trimethyl-12-(2,3,4,5,6-d_5-phenylamino)dodeca-2,6,10-trien-1-ol 12b.

A mixture of compound **11a** (737 mg, 2.52 mmol), MeOH (8 mL), K_2CO_3 (600 mg) and water (3 mL) was stirred overnight at rt. The resultant mixture was concentrated under reduced pressure to remove MeOH and extracted with CH_2Cl_2 (50 mL × 2). The combined organic phases were washed with brine, dried over $MgSO_4$ and concentrated under reduced pressure. The crude product was purified by silica gel column chromatography using 30% EtOAc/hexanes to give compound **12a** (508 mg, 81%). compound **11b** (420 mg) gave 282 mg of compound **12b** (76%).
d_5-AGOH **12a** ^{1}H NMR ($CDCl_3$) δ 1.64 (s, 3H), 1.65 (s, 3H), 2.03 (t, *J* = 8.0 Hz, 2H), 2.12–2.17 (m, 2H), 3.61 (s, 2H), 4.09 (d, *J* = 2.8 Hz, 2H), 5.32–5.40 (m, 2H)
HR mass *m/z* (M + H) 251.2159
d_5-AFOH **12b** ^{1}H NMR ($CDCl_3$) δ 1.58 (s, 3H), 1.62 (s, 3H), 1.65 (s, 3H), 1.95–2.08 (m, 4H), 2.09–2.18 (m, 4H), 3.60 (s, 2H), 4.18 (d, *J* = 4.2 Hz, 2H), 5.08 (t, *J* = 6.8 Hz, 1H), 5.38–5.41 (m, 2H)
HR mass *m/z* (M + H) 319.2792

Syntheses of (*R*)-methyl 2-amino-3-(((2*E*,6*E*)-3,7-dimethyl-8-(2,3,4,5,6-d_5-phenylamino)octa-2,6-dien-1-yl)thio)propanoate 14a and (*R*)-methyl 2-amino-3-(((2*E*,6*E*,10*E*)-3,7,11-trimethyl-12-(2,3,4,5,6-d_5-phenylamino)dodeca-2,6,10-trien-1-yl) thio)propanoate 14b

To a stirred solution of compound **12a** (200 mg, 0.8 mmol) and *N*,*N*-diisopropylethylamine (0.24 mL, 2.9 equiv. 2.3 mmol) in dry CH_3CN (5 mL) was added Ph_3PCl_2 (450 mg, 1.35 mmol, 1.7 equiv.) evenly over a 7-min period. After the final addition of Ph_3PCl_2, the reaction mixture was allowed to stir at 0°C for an additional 40 min and then cooled to −20°C. Then, a solution of L-cysteine methyl ester hydrochloride salt (542 mg, 3.2 mmol) in 7 M NH_3/MeOH (10 mL) was added and stirred for 5 h before warming to rt. The resultant reaction mixture was partitioned between Et_2O and water and extracted with Et_2O (50 mL × 4). The combined Et_2O extracts were then dried over $MgSO_4$, concentrated under reduced pressure and purified by silica gel chromatography using 1:2:8 ratio of *i*-PrOH, EtOAc and hexanes to obtain compound **14a** (38 mg, 13% in two steps). **12b** (100 mg) gave 15.9 mg of **14b** (12% in two steps).
14a ($CDCl_3$) δ 1.62 (s, 3H), 1.63 (s, 3H), 2.01–2.07 (m, 2H), 2.10–2.16 (m, 2H), 2.68 (dd, J = 7.6, 13.6 Hz, 1H), 2.87 (dd, *J* = 4.4,13.6 Hz, 1H), 3.09–3.19 (m, 2H), 3.60 (s, 2H), 3.64–3.67 (m, 1H), 3.71 (s, 3H), 5.16–5.23 (m, 1H), 5.33–5.37 (m, 1H)
LR mass *m/z* (M + 1) 368.3
14b ($CDCl_3$) δ 1.53 (s, 3H), 1.60 (s, 6H), 1.91–2.07 (m, 8H), 2.61 (dd, *J* = 8.0, 13.6 Hz, 1H), 2.81 (dd, *J* = 4.4,13.6 Hz, 1H), 3.06–3.18 (m, 1H), 3.56 (d, *J* = 5.6 Hz, 2H), 3.67 (s, 3H), 5.02 (t, *J* = 6.8 Hz, 1H), 5.16 (t, *J* = 8.0 Hz, 1H), 5.33 (t, *J* = 7.6 Hz, 1H)
LR mass *m/z* (M + H) 436.2

Syntheses of (*R*)-2-amino-3-(((2*E*,6*E*)-3,7-dimethyl-8-(2,3,4,5,6-d_5-phenylamino)octa-2,6-dien-1-yl)thio)propanoic acid 15a and (*R*)-2-amino-3-(((2*E*,6*E*,10*E*)-3,7,11-trimethyl-12-(2,3,4,5,6-d_5-phenylamino)dodeca-2,6,10-trien-1-yl)thio) propanoic acid 15b

A mixture of compound **14a** (15 mg, 0.041 mmol), LiOH (10 mg, 0.42 mmol) in 1:1 ratio of isopropanol/water (1 mL) was stirred overnight. The pH of reaction mixture was adjusted to 5 using drops of 1 N HCl, filtered through a syringe Whatman filter (13 mm CD/X disposable polypropylene prefilter). This crude product solution was purified by RP-HPLC (C_{13} column) using CH_3CN/H_2O (0.1% TFA) to give compound **15a** (8 mg, 56%). **14b** (7.0 mg) gave 3.5 mg of compound **15b** (52%).
15a (CD_3OD) δ 1.65 (s, 3H), 1.67 (s, 3H), 2.03 (t, *J* = 7.2 Hz, 2H), 2.12–2.17 (m, 2H), 2.85 (dd, *J* = 8.8, 14.8 Hz, 1H), 3.26–3.29 (m, 3H), 3.70 (s, 2H), 4.01 (dd, *J* = 4.4, 8.8 Hz, 1H), 5.18 (t, *J* = 6.8 Hz, 1H), 5.42 (t, *J* = 6.8 Hz, 1H)
HR mass *m/z* (M + H) 354.2267
15b (CD_3OD) δ 1.49 (s, 3H), 1.61 (s, 6H), 1.87–2.07 (m, 8H), 2.80 (dd, *J* = 8.8, 14.8 Hz, 1H), 3.04 (dd, *J* = 4.4, 15.2 Hz, 1H), 3.10–3.15 (m, 1H), 3.68 (s, 2H), 3.98 (dd, *J* = 4.4, 8.8 Hz, 1H), 4.99 (t, *J* = 6.8 Hz, 1H), 5.15 (t, *J* = 7.6 Hz, 1H), 5.36 (t, *J* = 7.6 Hz, 1H)
HR mass *m/z* (M + H) 422.2889

Syntheses of (2*E*,6*E*)-3,7-dimethyl-8-(2,3,4,5,6-d_5-phenylamino)octa-2,6-dien-1-yl-diphosphate 16a and (2*E*,6*E*,10*E*)-3,7,11-trimethyl-12-(d_5-phenylamino)dodeca-2,6,10-trien-1-yl-diphosphate 16b

To a stirred solution of compound **11a** (192 mg, 0.77 mmol) in dry CH_3CN (10 mL) at 0°C was added Ph_3PBr_2 (324 mg, 0.77 mmol). After stirring for 4 h, $((n\text{-}Bu)_4N)_3HP_2O_7$ (2.96 g, 3.02 mmol) was added. Then, the resultant reaction mixture was stirred for 1 h and concentrated. The residue was washed with Et_2O (5 mL), the organic wash discarded and then purified as for compound **9** to give diphosphate **16a** as a white powder (37.9 mg, 11% in two steps). Compound **11b** (26 mg) gave 4.5 mg of compound **16b** (10% in two steps).

d_5-AGPP **16a** ^{1}H NMR (D_2O) δ 1.36 (s, 3H), 1.42 (s, 3H), 1.78–1.82 (m, 2H), 1.87–1.91 (m, 2H), 3.38 (s, 2H), 4.20 (t, J = 6.0 Hz, 2H), 5.15–5.20 (m, 2H)

^{31}P NMR (D_2O) δ −6.10(d, J = 22.0 Hz, 1P), −9.39 (d, J = 22.0 Hz, 1P)

HR mass m/z (M − H) 409.6127

d_5-AFPP **16b** ^{1}H NMR (D_2O) δ 1.39 (s, 3H), 1.41 (s, 3H), 1.53 (s, 3H), 1.80–1.96 (m, 8H), 3.46 (s, 2H), 4.28 (t, J = 6.8 Hz, 2H), 4.94 (t, J = 6.4 Hz, 1H), 5.20 (t, J = 6.4 Hz, 1H), 5.27 (t, J = 6.4 Hz, 1H)

^{31}P NMR (D_2O) δ −9.53 (d, J = 22.6 Hz, 1P), −6.80 (d, J = 22.6 Hz, 1P)

LR mass m/z (M − H) 477.3

SUPPORTING INFORMATION

The ^{1}H NMR, ^{13}C NMR, HR mass spectra of **5a-b**, ^{1}H, ^{13}P and HR mass spectra of **9a**, ^{1}H NMR, HR mass spectra of **13a–b**, ^{1}H, ^{13}P spectra of **16a–b**, and HR and LR mass spectrum of **16a** and **16b**, respectively, are available.

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Conflict of Interest

The authors did not report any conflict of interest.

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