Supporting Information

Broadening the utility of farnesyltransferase-catalyzed protein labeling using norbornene-tetrazine click chemistry

Shelby A. Auger[1], Sneha Venkatachalapathy[1], Kiall Francis G. Suazo[1], Yiao Wang[1], Alexander W. Sarkis[1], Kaitlyn Bernhagen[1], Katarzyna Justyna[1], Jonas V. Schaefer[2], James W. Wollack[3], Andreas Plückthun[2], Ling Li,[4] Mark D. Distefano[1]*

1. Department of Chemistry, University of Minnesota, Minneapolis, MN, 55455, USA
2. Department of Biochemistry, University of Zurich, Zurich, CH-8057, Switzerland
3. Department of Chemistry and Biochemistry, St. Catherine University, St. Paul MN, 55105, USA
4. Department of Experimental and Clinical Pharmacology, University of Minnesota, Minneapolis, MN, 55455, USA

Shelby A. Auger, auger054@umn.edu, 0000-0003-2873-2580

Sneha Venkatachalapathy, venka251@umn.edu, 0000-0002-6257-8874

Yiao Wang, wang6962@umn.edu, 0000-0002-0229-5768;

Kiall Francis Suazo, suazo003@umn.edu, 0000-0002-0803-8332

Alexander W. Sarkis, asarkis@brandeis.edu, 0009-0009-0554-0667

Kaitlyn Bernhagen, knbernhagen@gmail.com

Katarzyna Justyna, kjustyna@umn.edu, 0000-0003-4630-1581

Jonas V. Schaefer, Jonas.Schaefer@cslbehring.com, 0000-0002-8332-4371

James W. Wollack, jwwollack@gmail.com, 0000-0003-3124-721X

Andreas Plückthun, plueckthun@bioc.uzh.ch, 0000-0003-4191-5306

Ling Li, lil@umn.edu, 0000-0002-9245-7387

Mark D. Distefano, diste001@umn.edu, 0000-0002-2872-0259

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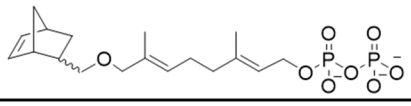
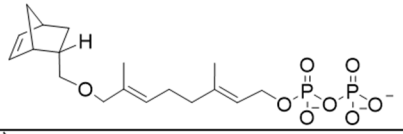
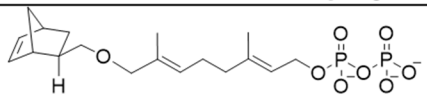
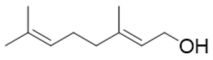
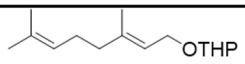
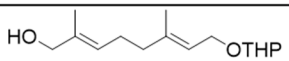
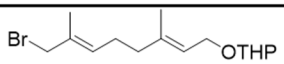
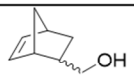
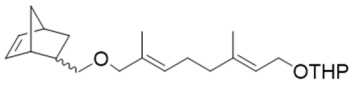
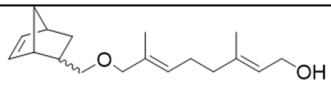
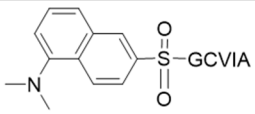
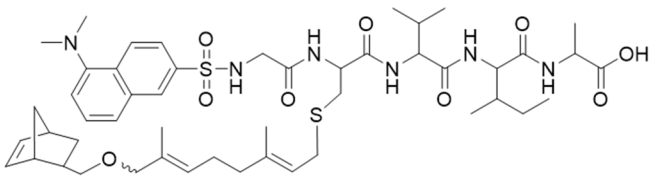
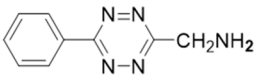
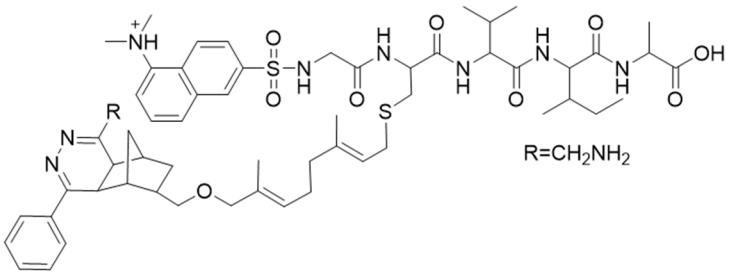
	**1; C10NorOPP,** Figure 1 A
	**1a;** *endo* C10NorOPP, Figure 2
	**1b;** *exo* C10NorOPP, Figure 2
	**2;** Geraniol, Scheme 1
	**3;** Scheme 1
	**4**; Scheme 1
	**5**; Scheme 1
	**6a** *endo* 5-norborene-2-methanol, Scheme 1 **6b** *exo* 5-norborene-2-methanol, scheme 1
	**7a** *endo,* Scheme 1 **7b** *exo,* Scheme 1
	**8a** *endo,* Scheme 1 **8b** *exo,* Scheme 1
	**9;** Ds-GCVIA, Figure S2
	**10;** Ds-GC[Nor]VIA, Figure S2
	**11;** Benzyl Amino Tetrazine, Figure S2



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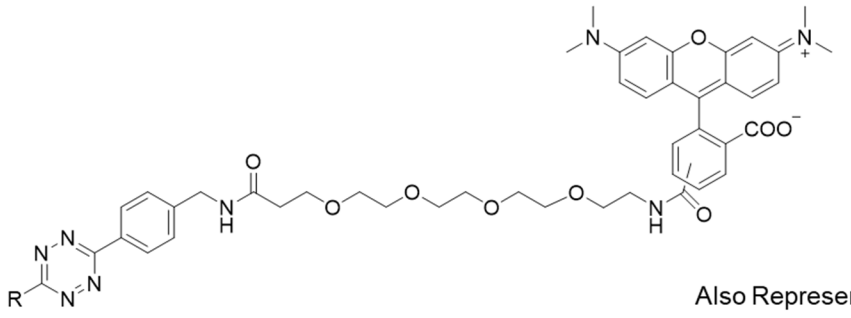

13; TAMRA-Tetrazine

13a;R=H,
13b;R=CH_3

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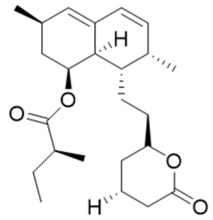
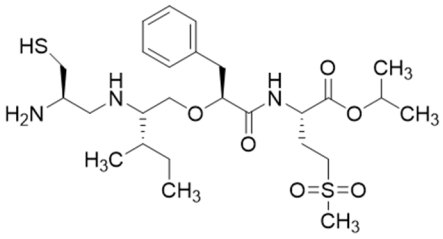
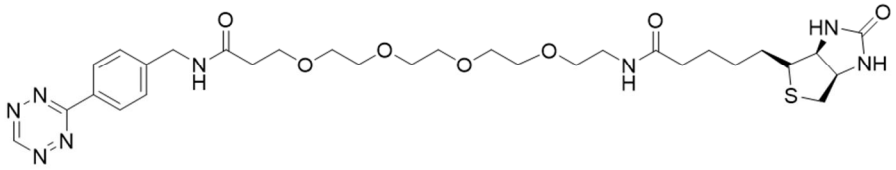


14; Lovastatin, Figure 4



15; FTI L-744,834, (FTI) Figure 4



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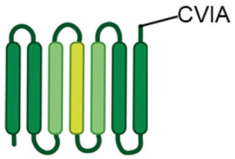
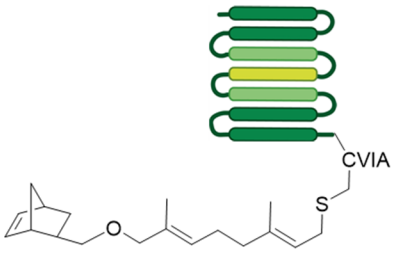
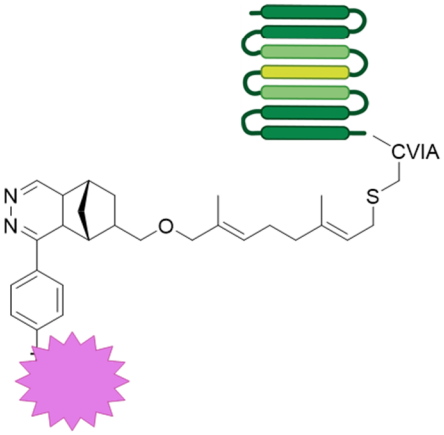
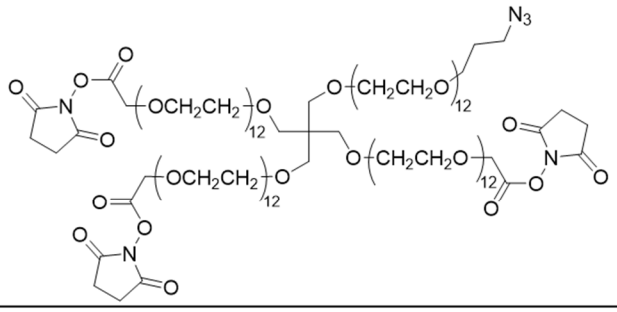
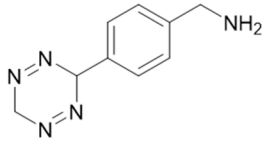
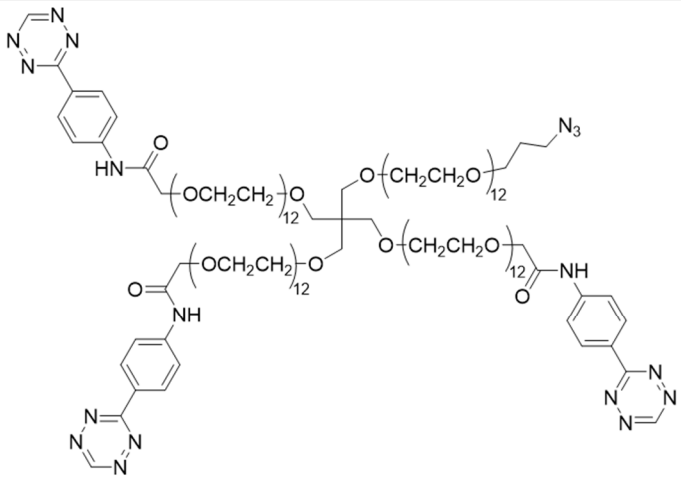


17; D1, anti-EpCAM DARPin Protein Ac2-CVIA , Figure 5

	**18;** D1-Nor, Figure 5
	**19;** D1-TAMRA, Figure 5
	**20**; 4-PEG-NHS$_3$-Az
	**21**; Tetrazine Amine



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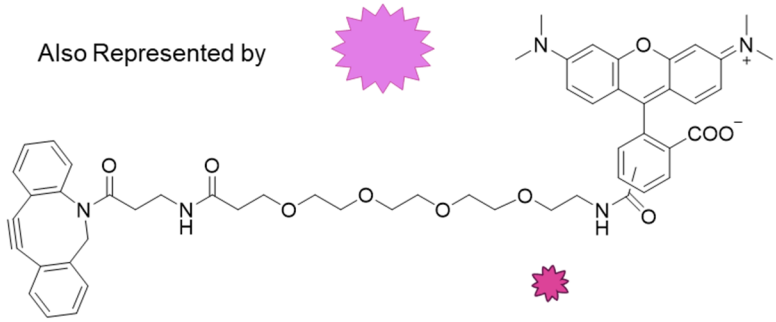

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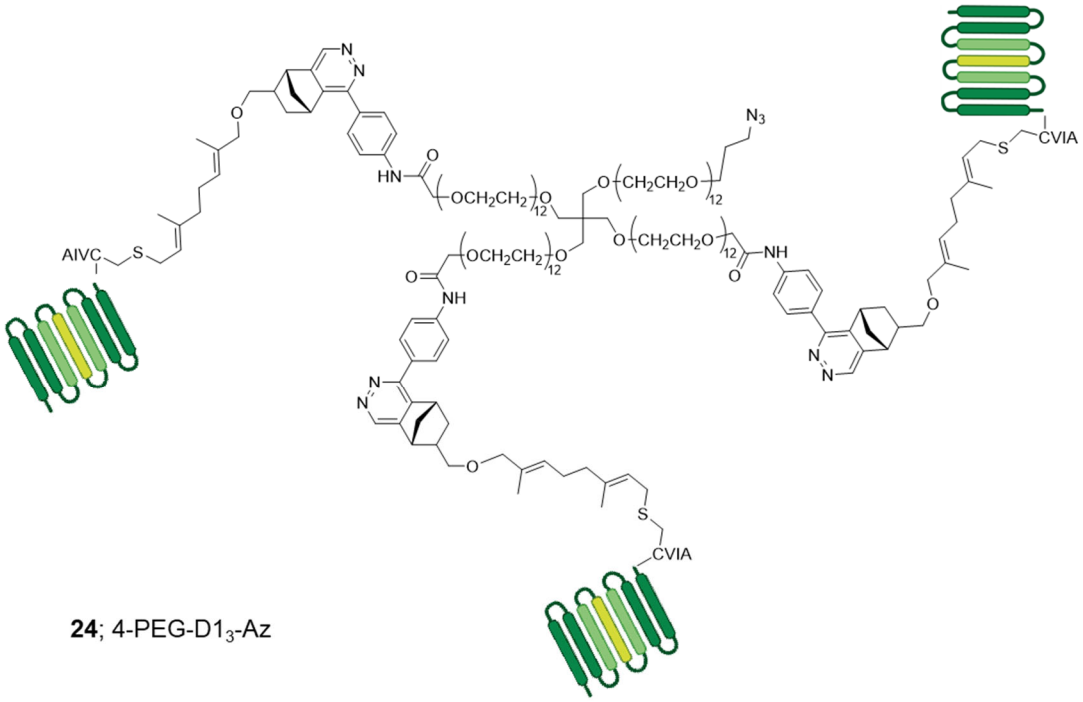

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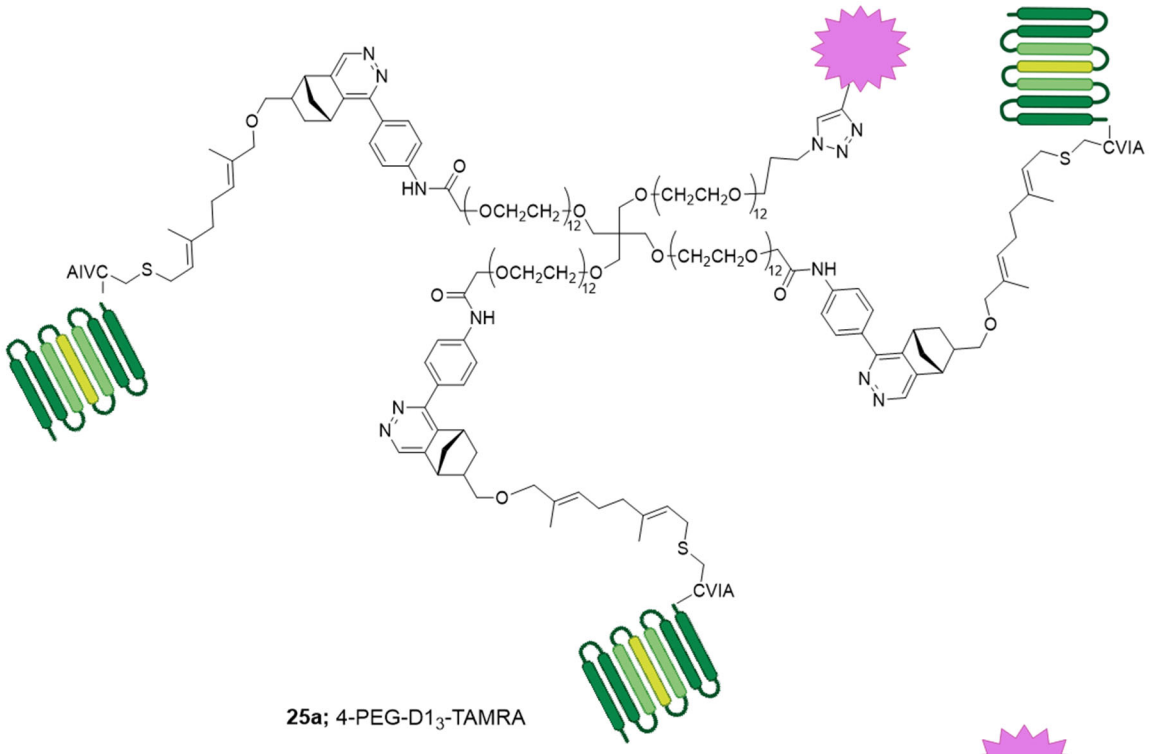

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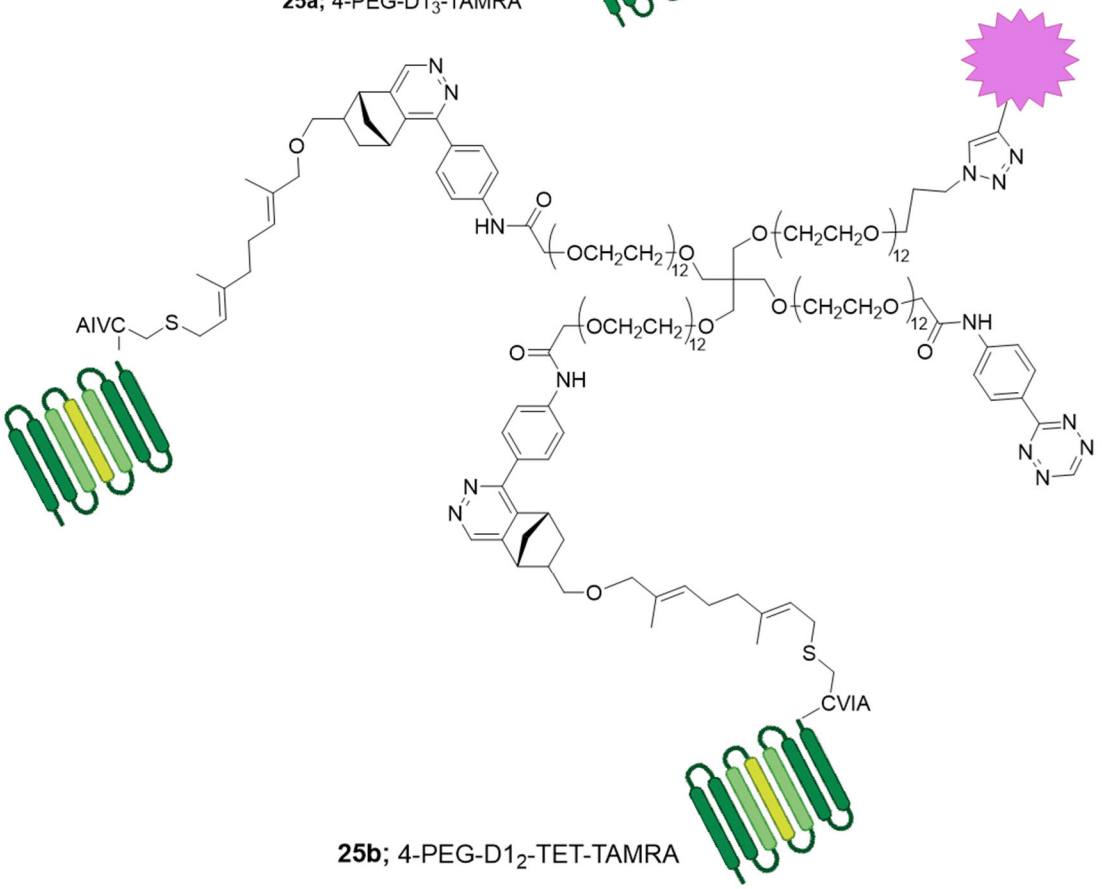

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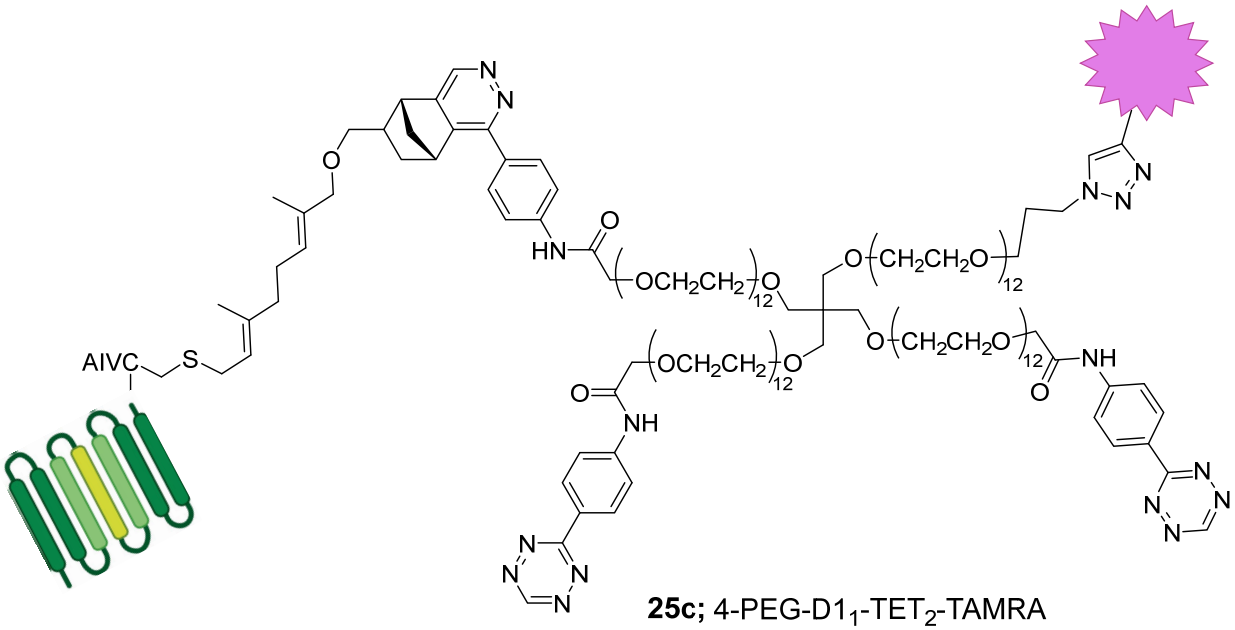


25c; 4-PEG-D1$_1$-TET$_2$-TAMRA

Experimental Methods

Purchased reagents were used without further purification. HeLa Cells were obtained Dr. E. Wattenberg at University of Minnesota. Immortalized Astrocyte Cells were obtained from Dr. L. Li at the University of Minnesota. COS7 cells were purchased from ATCC. MCF7 cells were obtained from Dr. B. Hakel at the University of Minnesota.

(E)-2-((3,7-dimethylocta-2,6-dien-1-yl)oxy)tetrahydro-2H-pyran (3): Compound **3** was prepared using a previously published procedure.[1] In brief, geraniol (**2**, 6.0 g, 38 mmol, Sigma Aldrich), Pyridinium p-toluenesulfonate (PPTs, 0.47 g, 1.9 mmol, Sigma Aldrich), and 3,4-dihydro-2H-pyran (5.0 mL, 60 mmol, Aldrich) were combined in CH_2Cl_2 (150 mL) and stirred overnight. After concentration, the solution was extracted with Et_2O (60 mL), washed with brine (3 x 60 mL), dried with $MgSO_4$ and concentrated *in vacuo* to yield **3** (20 g, >99% yield).as a clear oil. ^{1}H-NMR (400 MHz, $CDCl_3$, JOEL) δ ppm 5.36 (t, *J* = 6.8, 1H), 5.09 (t, *J* = 5.4, 1H), 4.6 (s,1H), 4.23 (q, *J* = 6.5, 1H), 4.08-3.99 (m, 1H), 3.89 (br.t, *J* = 9.4, 1H), 3.55-3.47 (m, 1H), 2.16-2.00 (m, 4H), 1.90-1.79 (m, 1H), 1.77-1.65 (m, 6H), 1.64-1.47 (m, 8H).

(2E,6E)-2,6-dimethyl-8-((tetrahydro-2H-pyran-2-yl)oxy)octa-2,6-dien-1-ol (4):
Compound **4** was prepared using a previously published procedure.[1] Briefly, THP-protected geraniol (**3**, 9.2 g, 38.8 mmol) was dissolved in CH_2Cl_2 (20 mL). Luperox tert-butyl hydroperoxide (*t*-BuOOH,10.5 g, 117 mmol, Aldrich), SeO_2 (0.56 g, 5.0 mmol, Sigma Aldrich), and salicylic acid (0.64 g, 5.0 mmol, Sigma Aldrich) were added and stirred overnight. The reaction was quenched with satd. $NaHCO_3$ (20 mL) and extracted with EtOAc (2 x 25 mL). The combined organic layers were washed with brine (25 mL), dried with $MgSO_4$ and concentrated *in vacuo*. The resulting oil was then dissolved in CH_3OH (20 mL), at 0 ^{o}C. $NaBH_4$ (1.5 g, 0.04 mmol) was then added and allowed to stir for 4 h. The reaction was quenched with H_2O (20 mL) and extracted with EtOAc (2 x 25 mL). The combined organic layers were washed with brine (25 mL) and dried with $MgSO_4$. The resulting crude material was purified by column chromatography (SiO_2, 2:5, EtOAc:hexanes, v/v) to yield **4** (5.6 g, 20 mmol) in 61% yield as a pale yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 5.35 (q, *J* = 6.6, 2H), 4.61 (s, 1H), 4.22 (q, *J* = 6.5, 1H), 4.05-3.99 (m, 1H), 3.97(s, 2H), 3.92-3.83 (m, 1H), 3.54-3.46 (m, 1H), 2.16 (q, *J* = 6.9, 2H), 2.06 (t, *J* = 2.1, 2H), 1.88-1.76 (m, 2H), 1.75-1.61 (m, 6H), 1.60-1.47 (m, 6H).

2-(((2E,6E)-8-bromo-3,7-dimethylocta-2,6-dien-1-yl)oxy)tetrahydro-2H-pyran (5): N-bromosuccinimide (NBS,1.38 g, 7.2 mmol, Acros Organics) was dissolved in anhydrous CH_2Cl_2 (10 mL) and cooled to -40 ^{o}C in dry ice bath made up of 60% $C_6H_6O_2$ in C_2H_6OH under N_2 for 30 min. Dimethyl sulfide (0.56 g, 9.0 mmol, Aldrich) was added dropwise over 30 mins and the reaction was stirred for 1 h at -40 ^{o}C. Next, the dry ice bath was removed, and the reaction mixture was allowed to warm to 0 ^{o}C over 1 h in an ice water bath followed by additional stirring at 0 ^{o}C for 30 min. The solution was then cooled to -40 ^{o}C by returning it to the dry ice bath and **4** (1.5 g, 5.9 mmol) was added dropwise. The reaction was maintained at -40 ^{o}C for 1 h and then allowed to warm to rt over 2 h. The reaction was monitored with TLC (8:2 Hex:EtOAc), quenched with $NaHCO_3$ (15 mL), and extracted with EtOAc (3 x 20 mL) and the combined organic layers were dried with

$MgSO_4$. The solution was purified via column chromatography (SiO_2, 9:1 Hexanes:EtOAc, v/v) yielding **5** (1.33 g, 4.4 mmol) in 66% yield as a yellow oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 5.59 (t, J = 7.0, 1H), 5.37 (t, J = 7.0, 1H), 4.63 (t, J = 3.1, 1H), 4.25 (dd, J = 6.4, J = 11.6, 1H), 4.03 (dd, J = 7.2, J = 11.6, 1H), 3.97 (s, 1H), 3.86-3.94 (m, 1H), 3.48-3.57 (m, 1H), 2.22-2.12 (m, 2H), 2.1-2.0 (m, 2H), 1.49-1.89 (m, 6H), 1.76 (s, 3H), 1.68 (s, 3H); ^{13}C-NMR, (128 MHz, $CDCl_3$,) δ ppm: 139.68, 131.96, 127.25, 121.02, 120.61, 97.81, 97.45, 72.68, 63.45, 62.08, 61.96, 39.01, 30.47, 26.33, 25.31, 19.43, 19.34, 16.22, 14.71, 14.00, 13.85; ^{13}C DEPT-135 NMR, (128 MHz, $CDCl_3$) δ ppm: 127.46, 120.81, 97.88, 97.45, 72.89, 63.66, 62.18, 39.21, 30.68, 26.05, 25.51, 19.63, 16.42, 14.06; HRMS (ESI), *m/z* for $[M-Br+OH+Na]^+$: Calcd. for $[NaC_{15}H_{26}O_3]$; 277.1785, Found 277.1788.

(±)-(2E,6E)-8-(((1S,2S,4S)-bicyclo[2.2.1]hept-5-en-2-yl)methoxy)-3,7-dimethylocta-2,6-dien-1-yl-diphosphate (7a) *and* (±)-(2E,6E)-8-(((1S,2R,4S)-bicyclo[2.2.1]hept-5-en-2-yl)methoxy)-3,7-dimethylocta-2,6-dien-1-yl-diphosphate (7b): In a thick-walled glass pressure tube, 5-norborene-2-methanol (**6,** 0.11 g, 1.07 mmol, Aldrich) was dissolved in anhydrous THF (1 mL) and placed in ice bath. NaH, (0.048 g ,1.9 mmol, TCI) was added, the pressure tube was sealed, and the reaction mixture was stirred for 10 min. Next, **5** (0.285 g, 0.90 mmol) was added and the solution was refluxed at 80 °C for 6 days. Then the solution was diluted with H_2O (20 mL), extracted with EtOAc (3 x 20 mL), washed with brine (2 x 20 mL), dried with Na_2SO_4, concentrated, and purified using flash chromatography (SiO_2, 95:5 Hexanes:EtOAc, v/v) to yield 0.236 g of a clear colorless oil **7** (72.9 % yield).^{1}H-NMR (400 MHz, $CDCl_3$): δ ppm 6.09-6.16 (m, 1H *endo*, 0.2H *exo*), 6.04-6.08 (m, 0.2H *exo*), 5.90-5.96 (m, 1H *endo*), 5.42 (t, J = 7.4, 1H), 5.36 (t, J = 7.4, 1H), 4.16 (d, J = 6.9, 2H), 3.73-3.97 (m, 2H), 3.41 (m, 0.2H *exo*), 3.25 (t, J = 9.0, 0.2H *exo*), 3.09 (dd, J = 6.7, 9.8 , 0.1H *endo*) 2.95 (t, J = 9.0, 1H *endo*), 2.93 (s, 1H *endo*), 2.79 (s, 1H *endo*), 2.79 (s, 0.2H *exo*), 2.75 (s, 0.2H *exo*), 2.30-2.40 (m, 1H *endo*), 2.17 (t, J = 7.6, 2.4H), 2.07 (t, J = 7.6, 2H), 1.78-1.86 (m, 1H *endo*), 1.69 (s, 3H), 1.64 (s, 3H), 1.60-1.65 (m, 0.2H *exo*), 1.39-1.45 (m, 1H *endo*), 1.21-1.30 (m, 0.2H *exo*, 1H *endo*), 1.10 (dt, J = 11.7, 3.9, 0.2 H *exo*), 0.48 (ddd, J = 2.4, 4.6, 11.4, 1H *endo*);^{13}C NMR (128 MHz, $CDCl_3$) δ ppm;140.01(endo), 139.98 (exo), 137.19 (*endo*), 136.77(*exo*), 136.71(*exo*), 132.43 (*endo*), 128.73 (*exo*), 128.58 (*endo*), 121.05, 97.98, 97.84, 74.62 (*exo*), 74.59(*endo*), 73.47 (*endo*), 73.35 (*exo*), 63.77, 62.44, 62.42, 49.54, 45.13 (*endo*), 45.12(*exo*), 44.11, 43.87 (*endo*), 43.67 (exo), 39.36 (*exo*), 39.01(*endo*), 38.92, 38.91, 29.90, 29.87, 29.31 (*exo*), 29.28 (*endo*),26.10, 25.63, 20.30,16.55,16.53, 13.97, 13.93; HRMS (ESI), *m/z*: $[M+H]^+$: Calcd. for $[C_{23}H_{37}O_3]$ 361.2737, Found 361.2732.

(±)-(2E,6E)-8-(((1S,2S,4S)-bicyclo[2.2.1]hept-5-en-2-yl)methoxy)-3,7-dimethylocta-2,6-dien-1-ol (8a) *and* (±)-(2E,6E)-8-(((1S,2R,4S)-bicyclo[2.2.1]hept-5-en-2-yl)methoxy)-3,7-dimethylocta-2,6-dien-1-ol (8b): Compound **7** (91 mg, 0.25 mmol) and PPTs (13 mg, 0.052 mmol, Aldrich) were combined in EtOH (12 mL) and refluxed overnight at 60 ^{o}C. The reaction was then concentrated and purified using flash chromatography (SiO_2, 4:1 Hex: EtOAc, v/v) to yield 56 mg of a clear colorless oil **8** (80.1 % yield).^{1}H-NMR (400 MHz, $CDCl_3$): δ ppm 6.09-6.15 (m, 1H *endo*, 0.2H *exo*), 6.03-6.08 (m, 0.2H *exo*), 5.90-5.95 (m, 1H *endo*), 5.32-5.42 (m, 2H), 4.63 (t, J = 3.2, 1H), 4.25 (dd, J = 12.6, 6.8, 1H), 4.03 (dd, J = 12.6, 6.8, 1H), 3.74-3.94 (m, 3H), 3.48-3.56 (m, 1H), 3.37-3.44 (m, 0.2H *exo*), 3.21 (t, J= 9.0, 0.2H *exo*), 3.07 (dd, J = 6.9, 10.0, 1H *endo*), 2.95 (t,

J = 9.0, 1H *endo*), 2.93 (s, 1H *endo*), 2.79 (s, 1H *endo*), 2.79 (s, 0.2H *exo*), 2.76 (s, 0.2H *exo*), 2.29-2.39 (m, 1H *endo*), 2.17 (t, J = 7.6, 2H), 2.08 (t, J = 7.6, 2H), 1.75-1.90 (m, 2H*)*, 1.69 (s, 3H), 1.64 (s, 3H), 1.50-1.75 (m, 4H) 1.39-1.45 (m, 1H *endo*), 1.21-1.30 (m, 0.2H *exo*, 1H *endo*), 1.10 (dt, J = 11.7, 3.9, 0.2H *exo*), 0.48 (ddd, J = 2.4, 4.6, 11.4, 1H *endo*);^{13}C NMR**,** (128 MHz, Bruker, $CDCl_3$) δ 139.22, 137.19, 136.69, 132.66 (exo), 132.58 (endo),127.23 (exo), 123.81(endo), 74.44 (endo), 73.56 (exo), 59.40, 49.52, 45.11 (exo), 45.09 (endo), 44.08 (exo), 43.84 (endo), 42.28, 41.63, 39.20, 38.96 (endo), 38.87(exo), 29.86(endo), 29.26 (exo), 26.03,16.37, 13.99. HRMS (ESI), *m/z*: $[M+Na]^+$: Calcd. for $[C_{18}H_{28}O_2Na]$ 299.1987; Found 299.1989.

(±)-(2E,6E)-8-(((1S,2S,4S)-bicyclo[2.2.1]hept-5-en-2-yl)methoxy)-3,7-dimethylocta-2,6-dien-1-yl diphosphate (1a) *and* (±)-(2E,6E)-8-(((1S,2S,4S)-bicyclo[2.2.1]hept-5-en-2-yl)methoxy)-3,7-dimethylocta-2,6-dien-1-yl diphosphate (1b): CB_4,(37 mg, 0.11 mmol, Alfa Aesar) and PPh_3-polymer bound (55 mg, 0.17 mmol, Sigma Aldrich) were added to CH_2Cl_2 (2 mL) and stirred for 20 min in order to allow for the resin to swell. Then **8** (23 mg, 0.083 mmol) dissolved in 0.5 mL of CH_2Cl_2 was added and the mixture was gently stirred overnight. At this time the solution was filtered and CH_2Cl_2 (10 mL) used to wash the polymer beads and flask. The filtrate was concentrated and redissolved in CH_3CN (2 mL). The phosphorylating reagent (n-$Bu_4N)_3HP_2O_7$ (0.174 mg, 0.198 mmol, Sigma Aldrich) was added and the solution was stirred for 24 h. At this time the reaction mixture was concentrated to an oil followed by conversion to its ammonium form using Dowex® AG 50W-X8 exchange resin. For that, the resin (10 g) was packed into a 30 x 1.5 cm column, washed with 3 column volumes of H_2O and converted to its basic form upon washing with 3 column volumes of H_2O/NH_4OH (3:1, v/v). Next the column was equilibrated with buffer A (25 mM NH_4HCO_3/isopropanol (49:1, v/v)) until the pH of the flow-through was neutral. The above crude product was then dissolved in 20 mL of buffer A, and slowly loaded onto the column over 20 min with collection of the eluate. An additional 20 mL of buffer A was passed though the column and collected. The combined eluates were lyophilized to give a white powder which was purified using RP-HPLC (C18, 250 x 10 mm, UV detection 220 nm; flow rate 10 mL/min; gradient 0-40% B over 40 min; solvent A: 25 mM $NaHCO_3$, solvent B: CH_3CN). Fractions containing product (RT = 25 min) were lyophilized to yield 10.5 mg of phosphorylated product **1** (27.6 % yield). e^{1}H-NMR (400 MHz, $CDCl_3$): δ ppm 6.07-6.17 (m, 0.75H *endo*, 0.25H *exo*), 5.90-5.95 (m, 1H *endo*), 3.38-5.47 (m, 2H), 4.42 (t, J = 6.9, 1H), 3.78-3.92 (m, 2H), 3.41-3.47 (m, 0.2H *exo*), 3.33 (dd, J = 9.0, 10.0, 0.2H *exo*) 3.10 (dd, J = 6.8, 10.0, 1H *endo*), 2.95 (t, J = 9.2, 1H *endo*), 2.83 (s, 1H *endo*), 2.79 (s, 1H *exo*), 2.76 (s, 1H *endo*), 2.64 (s, 0.2H *exo*), 2.23-2.33 (m, 1H *endo*), 2.17 (t, J = 7.2, 2H), 2.09 (t, J = 7.2, 2H), 1.74-1.82 (m, 1H *endo*), 1.68 (s, 3H), 1.60 (s, 3H), 1.56-1.61 (m, 0.2H, *exo*), 1.30-1.35 (m, 1H *endo*), 1.10-1.40 (m, 0.2H *exo*, 1H *endo*), 1.07 (dt, J = 11.7, 3.9, 0.2H *exo*), 0.48 (ddd, J = 2.4, 4.6, 11.4, 1H *endo*); ^{31}P NMR (121 MHz, D_2O) δ ppm; -6.45 (d, J = 12.1), -10.22 (d, J = 12.1); HRMS (ESI), *m/z*: Calcd. for $[C_{18}H_{30}O_8P_3]$ $[M]^-$ 435.1421; Found 435.1422.

Docking analysis of norbornene analogue C10NorOPP with rFTase: The structure of **1** in its di-anionic form was created in ChemDraw, saved as an MDL file, imported into Maestro (Schrödinger) and prepared for docking using the OPLS3 force field with a target pH of 7.0 ± 2.0. Similarly, the PDB file 1JCR containing the coordinates of rat

farnesyltransferase in complex with a non-substrate tetrapeptide inhibitor CVFM and FPP was prepared for docking in Maestro with a target pH of 7.0 ± 2.0 and optimized by removing any water molecules with less than 3 H-bonds to non-waters and minimized using the OPLS3 force field. A receptor grid around the bound FPP was created with a scaling factor of 1.0 and a partial charge cutoff of 0.25. Glide was then used to dock **1** into the protein using the extra precision mode with flexible ligand sampling and the top 20 poses recorded in the output file and viewed in Pymol. Since **1** is a mixture of *endo* and *exo* diastereomers (**1a** and **1b**), each structure was docked separately.

Enzymatic modification of *N*-Dansyl-GCVIA (9) with C10NorOPP to generate N-Dansyl-GC[C10-Nor]VIA (10). A 20.0 mL enzymatic reaction containing (all concentrations listed are final concentrations) 50 mM Tris·HCl, pH 7.5, 10 mM $MgCl_2$, 10 μM $ZnCl_2$, 15 mM DTT, 2.4 μM N-Dansyl-GCVIA (**9**), 10 μg yPFTase, and 6.5 μM C10NorOPP was performed at 30ºC in a water bath. Prior to initiating the reaction, all reagents except C10NorOPP and enzyme were incubated for 45 min at rt to ensure complete disulfide bond reduction. After this preincubation, C10NorOPP and enzyme were added and the reaction was allowed to proceed for 2 h. LC-MS analysis indicated only traces of unmodified N-Dansyl-GCVIA (**9**) remaining (solvent A = 0.1% HCO_2H in H_2O, solvent B = 0.1% HCO_2H in CH_3CN; gradient: 2% B for 1.5 min, 2 to 5% B over 1.5 min, 5 to 30% B over 11 min; t_R of N-Dansyl-GCVIA (**9**) was 5.9 min, LC-MS (ESI) *m/z*: $[M+H]^+$ Calcd for **9** 695.3; Found 695.2.The t_R of **10** was 13.3 min. LC-MS (ESI) *m/z*: $[M+2H]^{2+}$ Calcd for **10** 477.2; Found 477.0.
To isolate the enzymatic reaction product, a small reversed-phase cartridge was employed. A Sep-Pak C18 plus long cartridge (Waters, # WAT023635) was conditioned with 10 mL of 0.1% TFA in H_2O and 18.0 mL of the reaction mixture was passed through the column over 15 min. The column was washed with 10 mL of 0.1% TFA in H_2O, and the alkylated peptide was eluted using a step gradient of 0 to 80% D (1.5 mL fractions, solvent C = 0.1% TFA in H_2O, solvent D = 0.1% TFA in CH_3CN) using 10% steps (10%, 20%, 30% etc.) followed by three 1.5 mL fractions of 100% B. The product eluted in the 70% D, 80% D, and the first two 100% D fractions. These fractions fluoresced when irradiated with a handheld UV-lamp and were shown to contain product, using the LC-MS method reported above. Product-containing fractions were then lyophilized, dissolved into 100 μL of CH_3OH, and quantified using the extinction coefficient of the dansyl fluorophore (4,300 $L•mol^{-1}•cm^{-1}$ in CH_3OH). This measurement indicated a yield of 11.1 nmol of enzymatically modified product, N-Dansyl-GC[Nor]VIA, **10**, (26% yield).

Tetrazine ligation with 10 to produce 12. In a 1.5 mL microcentrifuge tube, N-Dansyl-GC[Nor]VIA (**10**, 4.6 μL of a 43 μM solution in CH_3OH) and benzyl amino tetrazine (**11**, 0.3 μL of a 10 mM solution dissolved in DMSO) were combined with 35 μL of 10 mM NaH_2PO_4 (pH 9.0) yielding final concentrations of peptide and benzyl amino tetrazine of 5 μM and 75 μM, respectively. This solution (40 μL) was then stirred in the dark using a small stir bar. After 2 h, both starting material and product were present in the mixture as determined by LC-MS analysis (solvent A = 0.1% HCO_2H in H_2O, solvent B = 0.1% HCO_2H in CH_3CN; gradient: 2% B for 1.5 min, 2 to 5% B over 1.5 min, 5 to 30% B over 11 min. The t_R for the ligated product (**12**) was 8.2 min. LC-MS (ESI) *m/z*: $[M+3H]^{3+}$ Calcd.

for **12** 371.5; Found 371.2. After stirring overnight (20 h) only ligated product (**12**) was observed upon further LC-MS analysis.

General cell culture and metabolic labeling. HeLa cells (1.5 x 10^6 cells/mL seeding density) were seeded in a 100-mm dishes with 10 mL of DMEM (Gibco) media containing 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Gibco). After overnight growth, the media was replaced with 5 mL of fresh media followed by appropriate treatment and subsequent growth for 24 h under 5% CO_2 at 37°C. For the *in vitro* experiments, HeLa cells were harvested through cell scraping. For the metabolic labeling experiments, HeLa cells were pre-treated with lovastatin (Cayman Chemical, final concentration: 25 μM or 10 μM) and in some cases in combination with L-744,834 (FTI, final concentration: 20 μM), for 6 h followed by treatment with either FPP (4 μL, 25 mM in 25 mM NH_4CHO_3 buffer, final concentration: 25 μM) or C10NorOPP (**1**, 3.4 μL, 14.8 mM in 25 mM NH_4CHO_3 buffer, final concentration: 10 μM) and grown for 24 h. Cells were collected by scraping and centrifugation and stored at -80°C until further use. Metabolically labeled cells were suspended in PBS (10 mM Na_3PO_4, pH 7.4, 137 mM NaCl, 2.7 mM KCl) containing 0.22 units/μL benzonase nuclease (final concentration, Sigma-Aldrich), 1.5% v/v protease inhibitor cocktail (Sigma-Aldrich), and 1% SDS. Cells were lysed by sonication on ice for 6-8 times of 2-sec pulses at 10-sec intervals. Protein concentrations were assayed using a BCA method and 100 μg aliquots were prepared for tetrazine ligation and in-gel fluorescence analysis. COS7 cells or immortalized Astrocyte cells (1.0 x10^6 cells/mL seeding density) were also seeded on 100-mm dishes with 10 mL of the media described above. After 24 h of growth. media was removed with aspiration and 5 mL of fresh media was added. For experiments with lovastatin pretreatment, either 2 μL of 25 mM **14** in DMSO (final concentration: 10 μM) or 5 of 25 mM **14** in DMSO (final concentration: 25 μM) was added. Then, after 6 h of incubation at 37°C in 5% CO_2, 3.4 μL of 14.8 mM **1** in 25 mM NH_4CO_3 in H_2O (final concentration: 10 μM) was added and cells incubated for another 24 h. Cells were harvested in the same manner as described above. Cell pellets were resuspended in 300 μL of PBS, containing 0.26 μL of benzonase nuclease, 0.64 μL of 100 mM phenylmethylsulfonyl fluoride (PMSF, Sigma Aldrich), and 5 μL of protease inhibitor cocktail (Sigma Aldrich) Cells were then lysed as described above.

***In vitro* prenylation.** HeLa cells were pre-treated with lovastatin (**14,** 25 μM final concentration) for 6 h before harvest, and were then suspended in prenylation buffer (55 mM HEPES pH 7.2, 50 mM NaCl, 10 mM $MgCl_2$, 10 μM $ZnCl_2$, 5 mM dithiothreitol (DTT, GoldBio), 0.02% w/v octyl-D-glucopyranoside (OGP, Sigma-Aldrich) with 1.5% v/v protease inhibitor cocktail (Sigma-Aldrich) and 60 μM PMSF. Lysis was carried out as described above. Protein concentrations were determined using a Bradford assay and 200 μg of proteins were aliquoted. In vitro prenylation on lysates was performed in 100 μL volumes (2 μg/μL protein) with C10NorOPP (**1**, 6 mM, final concentration) and rFTase (100 nM, final concentration) in the presence or absence of GFP-CVIA (final concentration: 2.5 μM). Reactions were carried out at 37°C for 4 h. Proteins were precipitated using a ProteoExtract precipitation kit (Calbiochem) following the manufacturer's protocol and redissolved in 200 μL of PBS + 1% SDS.

Tetrazine ligation and in-gel fluorescence analysis. Protein lysates from metabolic labeling or in vitro prenylation (1 µg/µL protein) were subjected to tetrazine ligation with TAMRA-PEG4-Tetrazine (TAMRA-Tetrazine, **13a,** 50 µM, final concentration, BroadPharm) in the dark overnight at rt. Proteins were precipitated using a ProteoExtract kit and resuspended in 1X loading buffer containing 2% SDS (v/v), 10% glycerol (v/v), 0.02% bromophenol blue (v/v) in 50 mM Tris-HCl pH 6.8. Labeled protein samples were resolved on 12% SDS-PAGE gels and detection for TAMRA fluorescence was performed using a Typhoon FLA 9500 (GE Healthcare). Gel images were processed in ImageJ.

Enrichment of proteins labeled using C10NorOPP. HeLa Protein lysates (2 mg of protein in 1 mL) from cells treated with lovastatin and FPP or C10NorOPP (prepared as described above) were subjected to tetrazine ligation with Biotin-PEG4-Tetrazine (Biotin-Tetrazine (**16,** 75 µM final concentration, stock dissolved in DMSO, BroadPharm) in the dark overnight at rt. $CHCl_3$ precipitation was performed (1 mL $CHCl_3$, 4 mL CH_3OH, 3 mL H_2O) and the protein pellets were washed with 4 mL CH_3OH. The biotinylated proteins were dissolved in 1 mL of PBS + 1% SDS and incubated with 100 µL (settled beads) of pre-washed Neutravidin® agarose beads (Thermo Scientific) for 2 h at rt. Beads were washed with 1 mL volumes of the following: 1X PBS + 1% SDS (3x), 1 X PBS (1x), 8 M urea (3x), 50 mM $HN(CH_2CH_3)_3HCO_3$ (TEAB, 3x). Resin beads with the bound proteins were suspended in 100 µL of 50 mM TEAB and subjected to on-bead digestion with 250 ng of sequencing grade trypsin (Promega) overnight at 37°C. Reactions were quenched by adding HCO_2H (0.5% final concentration) for 15 min and tryptic peptides were collected using spin columns to remove the beads. Beads were washed with 0.5% HCO_2H in H_2O and the wash solution pooled with the collected peptides followed by lyophilization.

Proteomic sample preparation. Dried peptides were dissolved in 200 mM NH_4HCO_3 and assayed for concentration using a BCA assay. Each peptide sample was normalized to the same amount (10 µg) and then was spiked with 150 fmol yeast ADH1 standard and normalized to the same volume (30 uL of 200 mM NH_4HCO_3). Peptides were then fractionated using two layers of SDB-XC extraction disks (3M, 1.07 mm x 0.50 mm i.d.) held in a 200 µL pipette tip. The disks were preconditioned by an initial wash (40 µL of 80% CH_3CN in H_2O) followed by equilibration (40 µL of 200 mM NH_4HCO_3). Following sample application, peptides were eluted using 40 µL volumes of 7, 13, and 80% CH_3CN in 200 mM NH_4HCO_3.Each fraction was dried by lyophilization and dissolved in Buffer A (30 µL ,2% CH_3CN with 0.1% TFA in H_2O). For desalting, samples were loaded onto one layer of pre-conditioned SDB-XC extraction disk (preconditioned with 40 µL of Buffer B (80% CH_3CN in H_2O containing 0.1% TFA) and equilibrated with 40 µL of Buffer A). The extraction disks were washed with 40 µL of Buffer A, eluted with 40 µL Buffer B, dried by lyophilization, and dissolved in 30 µL of 0.1% HCO_2H in H_2O.

LC-MS/MS data acquisition. Tryptic peptides from each sample fraction were separated using an RSLC Nano System (Dionex) coupled to an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific). Samples were injected and separated at 300 nL/min using a reversed-phase column (40 cm, 75 µm i.d.) packed with ProntoSIL C18AQ 3 µm matrix (Bischoff) prepared in-house. Two separate gradients were performed, the first at 2% to 25% of CH_3CN with 0.1% HCO_2H in H_2O over 60 min followed by the second 25%

to 45% CH_3CN with 0.1% HCO_2H in H_2O over 20 mins. The eluted peptides were sprayed directly into the coupled orbitrap using a Nanospray Flex source (Thermo Fisher Scientific) set to collect single microscan events at 30,000 resolution over a 300-1500 *m/z* range. The automatic gain control (AGC) target was set at 5 x 10^5 and a max injection time (IT) of 50 ms. Dynamic exclusion was allowed for 90 s. The top 15 most intense ions were triggered for CID MS/MS fragmentation at 35% normalized collision energy (NCE) with a 3 *m/z* isolation window. MS2 acquisition was performed with 5 x 10^3 AGC target and 25 ms max IT.

Proteomic data processing. The acquired .raw files were analyzed using Sequest embedded in Proteome Discoverer version 1.4.1.14 against the non-redundant human database from Uniprot (ID UP000005640). The precursor and fragment mass tolerances (monoisotopic) were set to 10 ppm and 0.6 Da, respectively. Methionine oxidation was set as variable modification and peptides sequences from proteins were determined from full tryptic digestion with a maximum of four allowed missed cleavages. A decoy search was also performed. Results were exported as .msf files that were further analyzed using X! Tandem (2010.12.01.1) embedded in Scaffold version 4.5.1 (Proteome Software Inc., Portland, OR). Further variable modifications Glu to pyro-Glu of the N-terminus, ammonia-loss of the N-terminus, Gln to pyro-Glu of the N-terminus and oxidation of methionine were specified. Peptide and protein false discovery rates (FDR) were set at 1% and 5%, respectively. Identified proteins are accepted if they are at least greater than 95% probability and at least have 2 minimum peptides. Protein grouping was based on the principles of parsimony and proteins sharing peptide sequences were grouped into clusters. Quantitation was performed using spectral counting. Proteins with no spectral counts in a sample were imputed with a value of 1. Proteins with four or more imputations of 1 were removed from analysis. Fold-changes were calculated by taking the average ratio of counts obtained with C10NorOPP-treated samples divided by counts obtained with FPP-treated samples. Data was imported to Microsoft Excel. For analysis and comparison to previously published data, proteins groups, where multiple proteins are identified from the same spectral data, are separated (ungrouped) into separate entries with the same fold change. An example of this is that NRas and HRas, were grouped into one data point in the C10NorOPP, but NRas was a single point in the C15AlkOPP so to make fair comparisons across the datasets NRas and HRas were ungrouped into induvial data points with the same log_2 fold change, 2.2.

Enzymatic farnesylation of DARPins. Farnesylation reactions were performed in buffer containing 50 mM Tris·HCl, 20 mM KCl, 10 mM $MgCl_2$, 5 mM DTT and 10 μM $ZnCl_2$. The solution was preincubated on ice for 30 min including 2.5 μM DARPin Ac2_CVIA (**D1,17**) or E3_5-CVIA (**D4**)[2,3] to insure complete thiol reduction. Next, C10NorOPP in 25 mM NH_4CHO_3 was added to the solution (20 μM, final concentration), followed by the addition of yeast PFTase (400 nM, final concentration). The reaction was carried out in a water bath at 32ºC for 6 h. To remove excess isoprenoid analog, buffer exchange using PBS was conducted three times with Amicon ultrafiltration filters (10 kDa cut-off). For every round of buffer exchange, 14.5 mL of fresh PBS was added and concentrated down to 0.5 mL via centrifugation at 5000 x g for 30 min.

DARPin-Norbornene conjugation to TAMRA-Tetrazine. These IEDDA reactions were performed with 2.5 μM DARPin-Norbornene (**18**) and 15 eq of TAMRA-PEG4-methyltetrazine (TAMRA-Tetrazine, **13b**) at rt for 12 h on a rotary shaker. No catalyst was employed. A PD-10 desalting column (Fisher Scientific, Cat: 4500148) was used to remove excess **13b** from the reaction mixture. A sample of 18 μL of 5 μM DARPin-Nor (**18**) was used to further characterize the conjugate by LC-MS using an Orbitrap Elite Hybrid Mass Spectrometer equipped with Agilent, ZORBAX 300 SB-C3, 2.1 × 100mm, 1.8-Micron, Cat: 858750-909.

Flow cytometry analysis of DARPin-TAMRA (19) binding to cell surface EpCAM. A PBSA solution was prepared using PBS and 1 mg/mL BSA. MCF-7 cells were used as the $EpCAM^+$ cell line in this study. The cells were harvested and washed twice with 5 mL of PBSA. Then cells were resuspended in PBSA and aliquoted into Falcon™ Round-Bottom polystyrene test tubes (Cat: 08-771-23, 1 x 10^6 cells/mL, 500 μL per tube) and centrifuged to pellet the cells. The resuspended cell pellet was incubated with 500 nM of the indicated DARPin-TAMRA (D1-TAMRA (**19**) or D4-TAMRA) which were diluted in 100 μL of PBSA (PBS containing 1mg/mL of BSA) and incubated in the dark for 45 min at 4ºC followed by washing twice with 600 μL of PBSA before analysis. For the competition experiments, the cells were first incubated with 25 μM unlabeled D1 at 4ºC for 15 min. Experiments were conducted using a BD Fortessa X-20 flow cytometer and 1 x 10^4 cells were counted. Data analysis was performed with FlowJo software (v10).

Visualization of D1-TAMRA (19) binding and internalization to MCF-7 cells. MCF-7 cells (1 x 10^5) were seeded onto sterile glass coverslips and incubated for 24 h. The cells were then supplied with fresh DMEM containing 500 nM D1-TAMRA (**19**) or D4-TAMRA and incubated for 1 h in the dark at 4ºC or 37ºC. For the competition experiments, the cells were first incubated with 25 μM unlabeled D1 (**17**) at 4ºC for 15 min, followed by the incubation with 500 nM **19** for 1 h in the dark at 4ºC. After incubation, the cells were washed with 1 mL PBS (3 x 3 min) and then fixed with 4% paraformaldehyde, followed by washing once with 1 mL PBS. After washing with PBS, the coverslips were mounted on glass slides using ProLong Gold Antifade Mountant with DAPI (Fisher Scientific, Cat: P36941) and sealed with nail polish. Images were obtained using an Eclipse Ti-E Wide Field Deconvolution Inverted Microscope (Nikon Instruments, Inc.).

Preparation of 4-PEG-Tet$_3$-Az (22) from 4-PEG-NHS$_3$-Az(20): In a 1.5 mL microcentrifuge tube, 4-PEG-NHS_3-Az (**20**, 10 mg, 4.8 mmol, Creative PEGworks, Cat: CPW-4401) and tetrazine amine (**21**, 3.6 mg, 19 mmol, Click Chemistry Tools, Cat: 1130-25) were dissolved in anhydrous DMF (0.5 mL). Then triethylamine (3 mg, 28.6 mmol, Sigma Aldrich, Cat: 90340) was added and mixed thoroughly. The reaction was rotated at rt overnight followed by analysis via LC-MS on an Agilent 1200 series system (ChemStation Software, G1322A Degasser, G1312A binary pump, G1329A autosampler, G1315B diode array detector, 6130 quadrupole) equipped with a C18 column (Agilent ZORBAX 300-SB-C18, 5 μm, 4.6 x 250 mm). Separations were performed at a flow-rate of 1 mL/min. An H_2O/CH_3CN solvent system containing 0.1% TFA was used, consisting of solvent A (H_2O with 0.1% TFA) and solvent B (CH_3CN with 0.1% TFA). Samples were filtered through a 0.2 μm GHP filter before injecting into the instrument. Absorbance at

280 nm was recorded and used to confirm the addition of tetrazine to the polymer. The desired peak was purified via HPLC using an Agilent 1100 series system (ChemStation Software, G1312A binary pump, G1329A autosampler, G1315B diode array detector, Telodyne Foxy R1 fraction collector). HPLC purification was performed on a semipreparative scale (2 - 10 mg sample per injection, Agilent ZORBAX 300SB-C18 5 µm 9.4 × 250 mm) with a 4 mL/min flow-rate using the same Solvent A/Solvent B system described above. The gradient was as follows: 10 min hold at 1% B, 50 min ramp to 100% B, 10 min hold at 100% B (column wash),1 min ramp down to 1% B, 15 min hold at 1% B (equilibration). See Figure S12B.

Tetrazine Click Reaction with Nor-D1 (18) conjugate. 4-PEG-Tet_3-Az (**22**) reagent purified via HPLC was used to prepare a of 0.7 mM stock solution in DMSO. The polymeric reagent (**22**, 32 µM, 42 µL) was treated with 3 equiv. of farnesylated D1-Nor (95 µM, 858 µL) (**18)** in 1x PBS to perform the inverse electron demand Diels–Alder (IEDDA) reaction at 4°C overnight on a rotary shaker. Following that, the crude reaction mixture was carried forward for the Strain Promoted Azide Alkyne Click (SPAAC) reaction. For that purpose, TAMRA-PEG4-DBCO (**24**, 5 equiv, 2.8 µL, 50 mM stock in DMSO, BroadPharm, Cat: BP-22456) was added to crude **22,** prepared as described above and reacted at 4°C for 16 h.

Purification of 4-PEG-$D1_3$-TAMRA (25a) Conjugate. The crude SPAAC reaction mixture consisting of 4-PEG-$D1_3$-TAMRA (**25a**), 4-PEG-$D1_2$-Tet-TAMRA (**25b**), 4-PEG-D1-Tet_2-TAMRA (**25c**) as well as unreacted D1-Nor (**18**) and TAMRA-DBCO (**24**) reagent was subjected to Ni-NTA affinity chromatography purification using Knauer Azura FPLC including an AZURA® Pump P 6.1L pump system, KNAUER AZURA V 2.1S with 6-port multi-position valve head and a UV detector AZURA UV Detector UVD 2.1S. An A/B solvent system consisting of solvent A (PBS, 500 mM NaCl, 50 mM imidazole, 10% glycerol) and solvent B (PBS, 500 mM NaCl, 250 mM imidazole, 10% glycerol) was used. Samples were filtered through a 0.22 µm GHP filter before injecting into the instrument. A 1 mL HisPur™ Ni-NTA Chromatography Cartridge, (Thermo Scientific, Cat: 90098) was used. The gradient was as follows: 1-10 min hold at 0% solvent B with a flow rate of 0.1 mL/min, 2 min ramp to 35% solvent B with a flow rate of 1 mL/min (that flow rate was maintained for the rest of the profile), 63 min hold at 35% solvent B, 25 min ramp to 100% solvent B, 20 min hold at 100% solvent B, 1 min ramp down to 0% solvent A and a 10 min hold at 0% solvent A (equilibration). The fractions from 42 min to 75 min were pooled together to obtain Fraction 1, then samples eluting from 75 min to 100 min were pooled together to obtain Fraction 2. Those samples were analyzed by SDS PAGE with material from Fraction 2 being used for subsequent flow cytometry experiments.

Flow cytometry experiments with 4-PEG-$D1_3$-TAMRA (25a) Conjugate and D1-TAMRA (19): A PBSA solution consisting of PBS (Gibco, Cat: 10010023) and 1 mg/mL BSA was used for these experiments. MCF-7 cells were used as the $EpCAM^+$ cell line in this study. The cells were harvested and washed twice with 5 mL of PBSA. Then cells were resuspended in PBSA and aliquoted into 11 tubes (11 x 500 µL aliquots of $1x10^6$ cells/mL) were prepared and incubated with the following 10 concentrations of 4-PEG-$D1_3$-TAMRA (**25a**) or D1-TAMRA **(19)**: 125 nM, 62.5 nM, 31.2 nM, 15.6 nM, 7.8 nM, 3.9

nM, 1.9 nM, 0.9 nM, 0.4 nM, and 0.2 nM, which were serially diluted in PBSA in the dark and left for 45 min at 4°C. One tube containing no DARPin (0 nM **25a** or **19**) was used as a negative control. After incubation, the cells were washed with PBSA (2 x 1 mL) before analysis. Measurements were performed using a BD Fortessa X-20 flow cytometer at the University Flow Cytometry Resource (UFCR) and $1x10^4$ cells were counted. Data analysis was performed with FlowJo software (v10). For the affinity titration assays, data analysis and data visualization were performed in GraphPad Prism (v8). The data were fit using the equation $Y = F_{max} \bullet X/(K_d + X)$, where Y is the Mean Fluorescence Intensity value measure at a specific DARPin concentration, F_{max} is the maximum Mean Fluorescence Intensity, X is the [DARPin]; and K_d is binding constant. All K_d values derived include the standard error reported for these curve fits. Experiments were conducted in triplicate and data are presented as the mean ± standard deviation of three independent trials. The mean fluorescence intensity (MFI) obtained for the negative control (unstained sample) was subtracted from the MFI of each of the 10 samples from the affinity titration experiment. MFI data were converted to "Fraction Bound" values by dividing individual MFI values by the F_{max} value determined for the specific data set. Plots of "Fraction Bound" versus [Conjugate] were created for **25a** (4-PEG-D1$_3$-TAMRA) and **19** (D1-TAMRA) using these processed data.

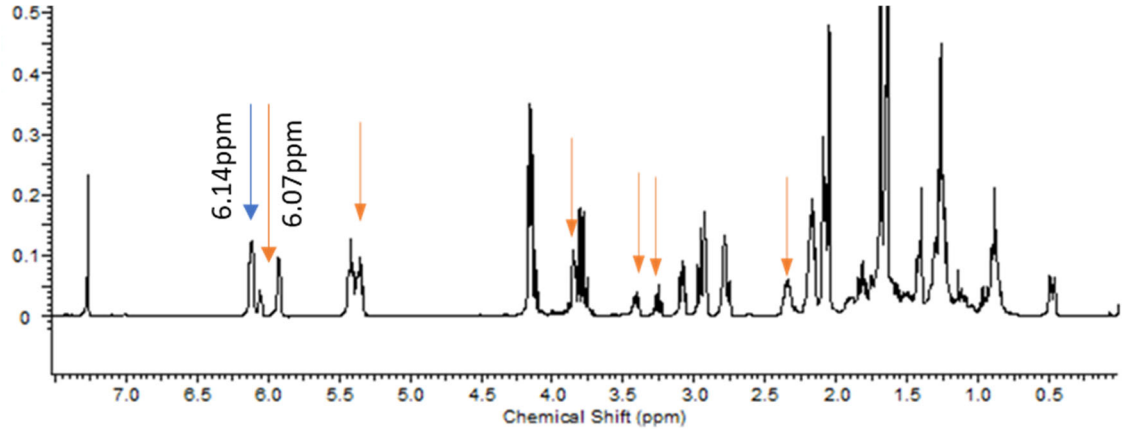


Figure S1: 1H-NMR of 7a/7b Peaks associated with endo and exo isomers.
Endo isomer peaks are indicated by orange arrows. The relative ratio of *endo vs. exo* was calculated using the integration values from the peak at 6.14 ppm, which corresponds to H-1 of the *endo* isomer (blue arrow) and the peak at 6.07 ppm, which corresponds to H-1 of the *exo* isomer.

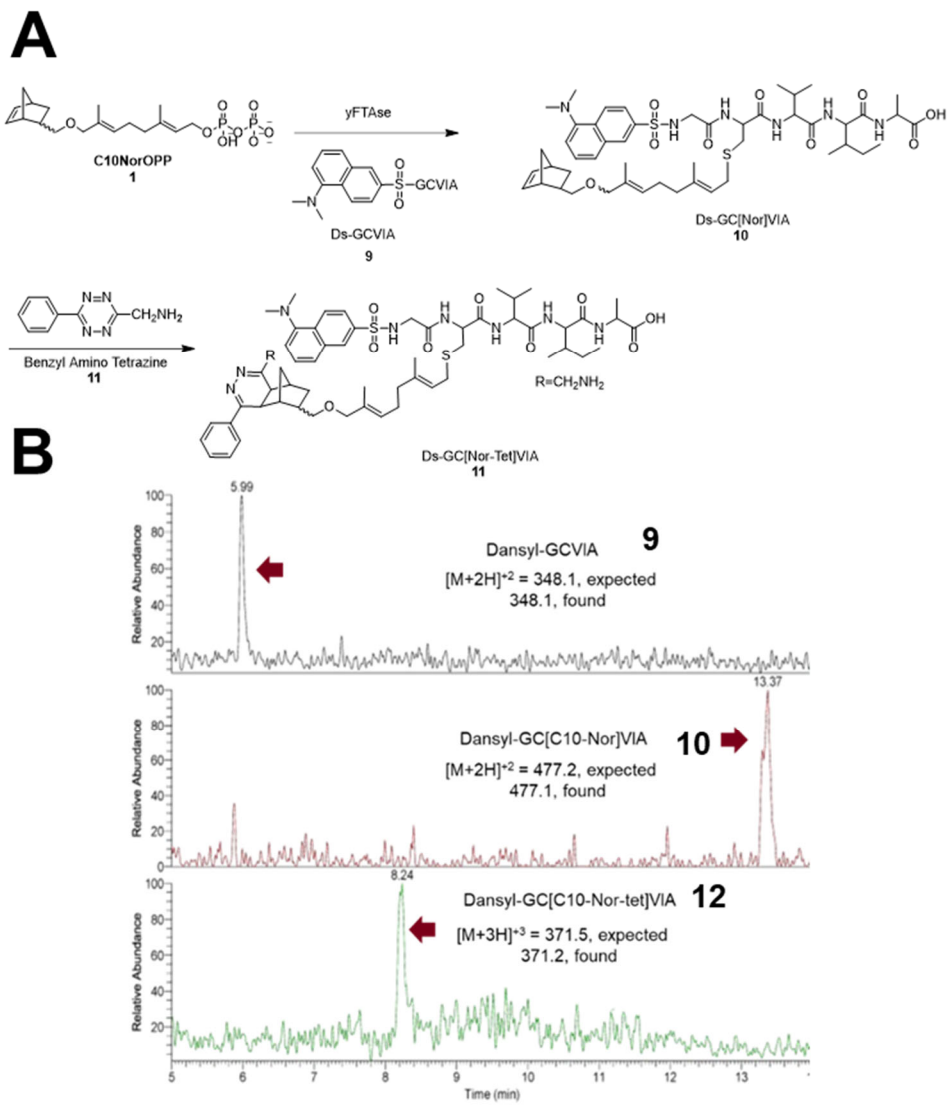


Figure S2: Confirmation of C10NorOPP (1) being a substrate for FTase through *in vitro* prenylation of Ds-GCVIA (9).
(A) Modification of Ds-GCVIA with C10NorOPP through the formation of a thioether bond on cysteine to generate Ds-GC[Nor]VIA (**10**). This enzymatic transfer was followed by the cycloaddition of benzyl amino tetrazine to produce Ds-GC[Nor-TET]VIA (**12**). These reactions were analyzed with LC-MS. (B) LC-MS analysis of *in vitro* prenylation reaction and subsequent tetrazine ligation. An *in vitro* reaction with Ds-GCVIA, a peptide substrate, and C10NorOPP with yFTase was analyzed by LC-MS. A subsequent tetrazine ligation reaction using the prenylated product was also analyzed. Top: Chromatogram of Ds-GCVIA t_R = 5.99 min). Middle: Chromatogram from in vitro prenylation reaction showing the formation of Ds-GC[Nor]VIA with a longer retention time characteristic of prenylation (t_R =13.37 min). Bottom: Chromatogram after reaction of Ds-GC[Nor]VIA with benzyl amino tetrazine to yield Ds-GC[Nor-TET]VIA (t_R = 8.24 min).

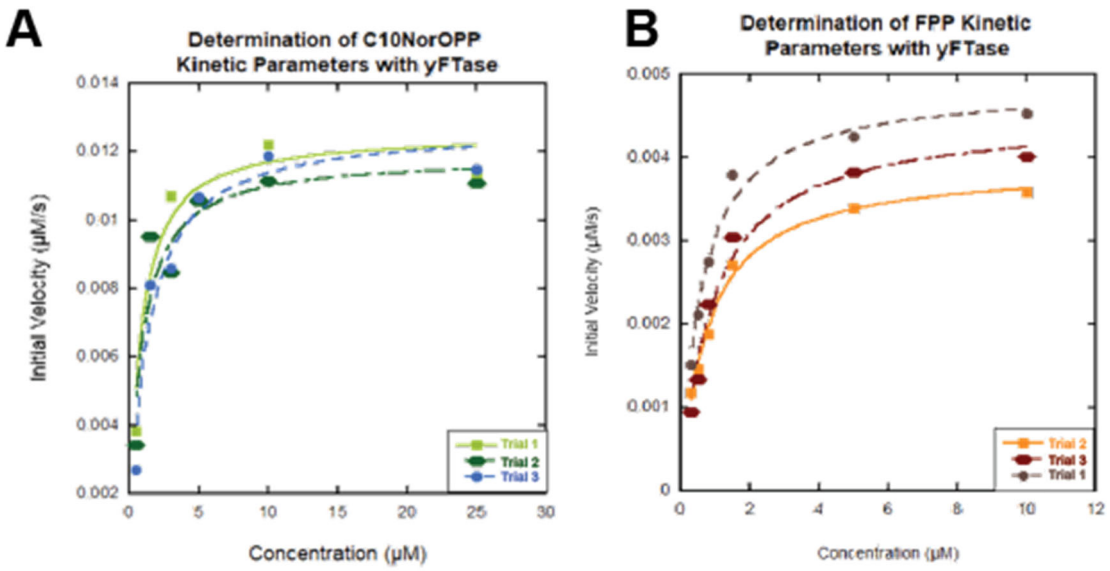


Figure S3: Kinetic analysis of yFTase-catalyzed prenylation of Ds-GCVIA using C10NorOPP (1) and FPP.
(A) Analysis of the prenylation Ds-GCVIA (**9**) with C10NorOPP (**1**). (B) Analysis of the prenylation Ds-GCVIA (**9**) with FPP. Rate measurements were performed at a single Ds-GCVIA concentration (2.4 μM) to yield apparent kinetic parameters. Three replicates for each were used to find the average values for K_m and V_{max}.

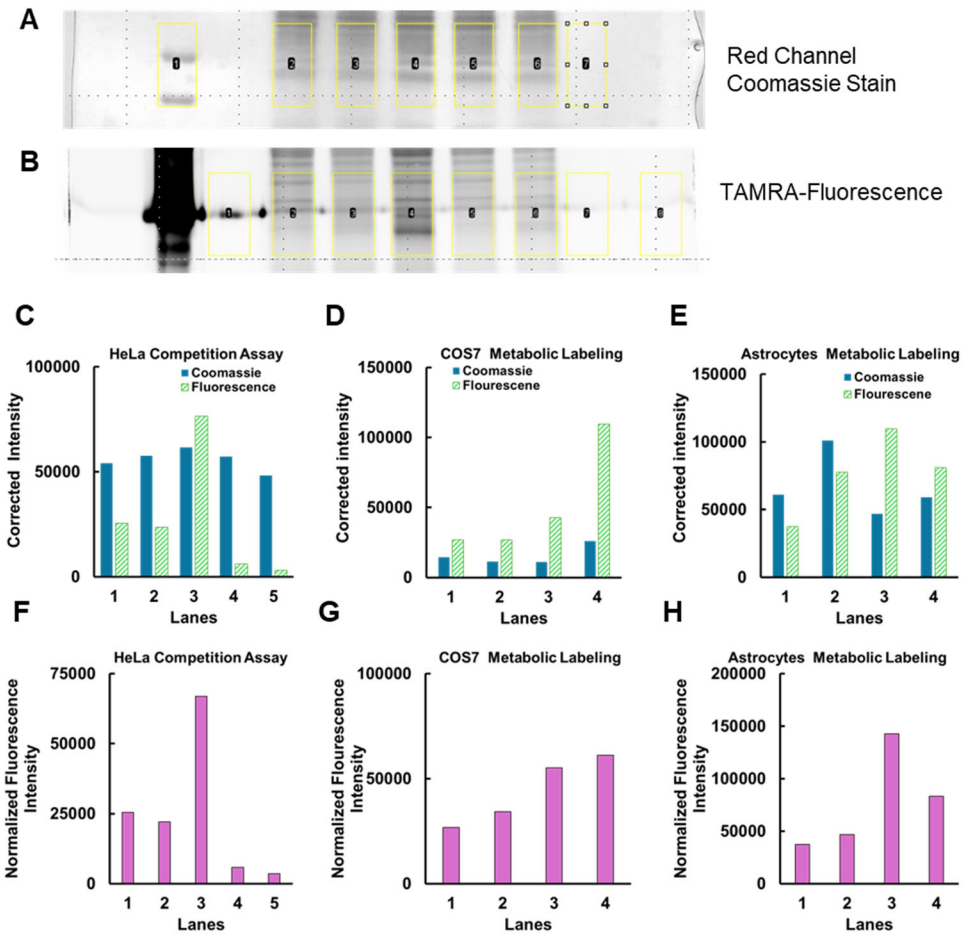


Figure S4: Quantification of normalized fluorescence intensity of metabolic labeling in different cell lines.

Example of the method used to quantify the intensity of the 25 kDa region of the red channel Coomassie blue stained gel (**A**) or TAMRA-Fluorescence visualized gel (**B**) measured with ImageJ. This example corresponds to the bottom panel (**A**) and the top panel (**B**) in Figure 4B. The yellow boxes indicate the regions measured in ImageJ; box 1 centered on the 25 kDa ladder band and boxes 7 or 8 are a measurement of the background intensity of the gel. The other yellow boxes measure the intensity of the lanes quantified in the plots. **C-E)** A plot of the background corrected quantification of the intensity of the total protein loading, visualized through total Coomassie stain, in blue and the background-corrected quantification of the TAMRA fluorescence intensity in green. **F-H)** The TAMRA fluorescence intensity normalized by protein loading for each gel, determined with the data plotted in **C-E.** The protein loading was normalized by dividing the Coomassie blue intensity of the untreated control (lane 1) by the background corrected Coomassie blue intensity. This resulting value quantifies the difference in protein loading between lanes. The background corrected fluorescence intensity was multiplied by the protein loading correction value to give the normalized fluorescence intensity.

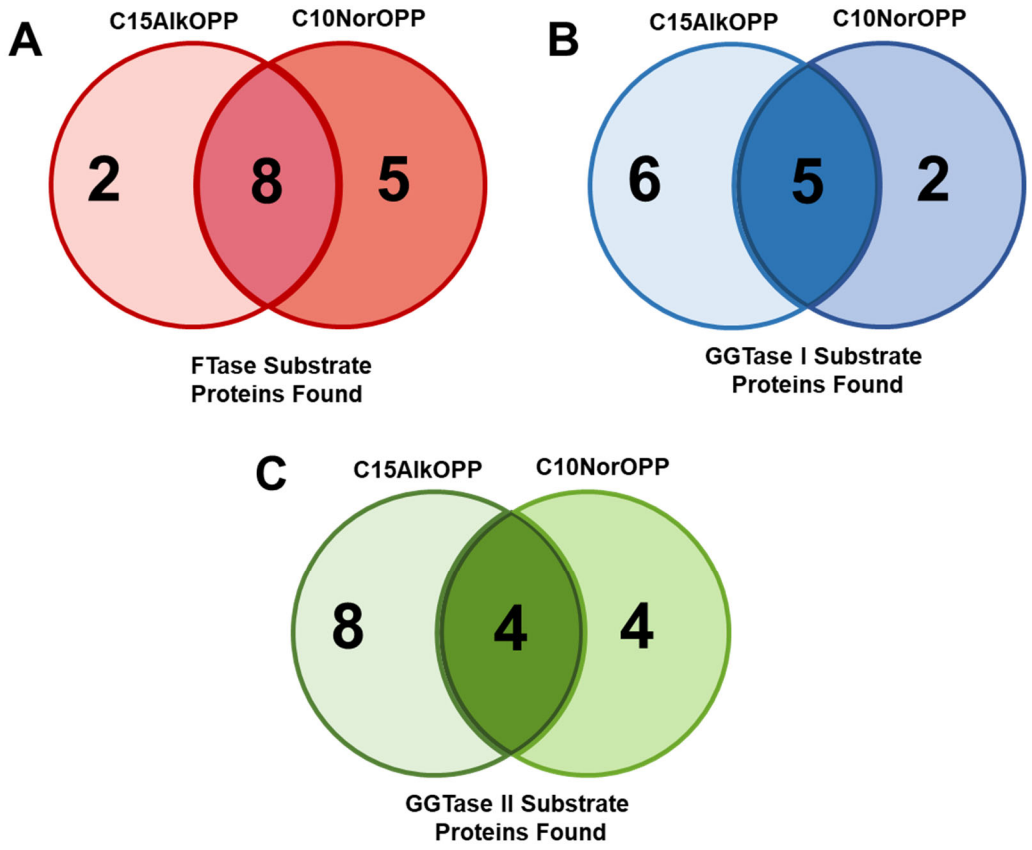


Figure S5: Comparisons of proteomic results from labeling in HeLa cells with C10NorOPP and with previously reported C15AlkOPP:
Ungrouped enriched prenylated proteins identities found with C10NorOPP and data collected with C15AlkOPP in total (A) subdivided by type of prenylation; (B) Red: Farnesylation, (C) Blue: Geranylgeranylation type I, (D) Green: Geranylgeranylation type II.

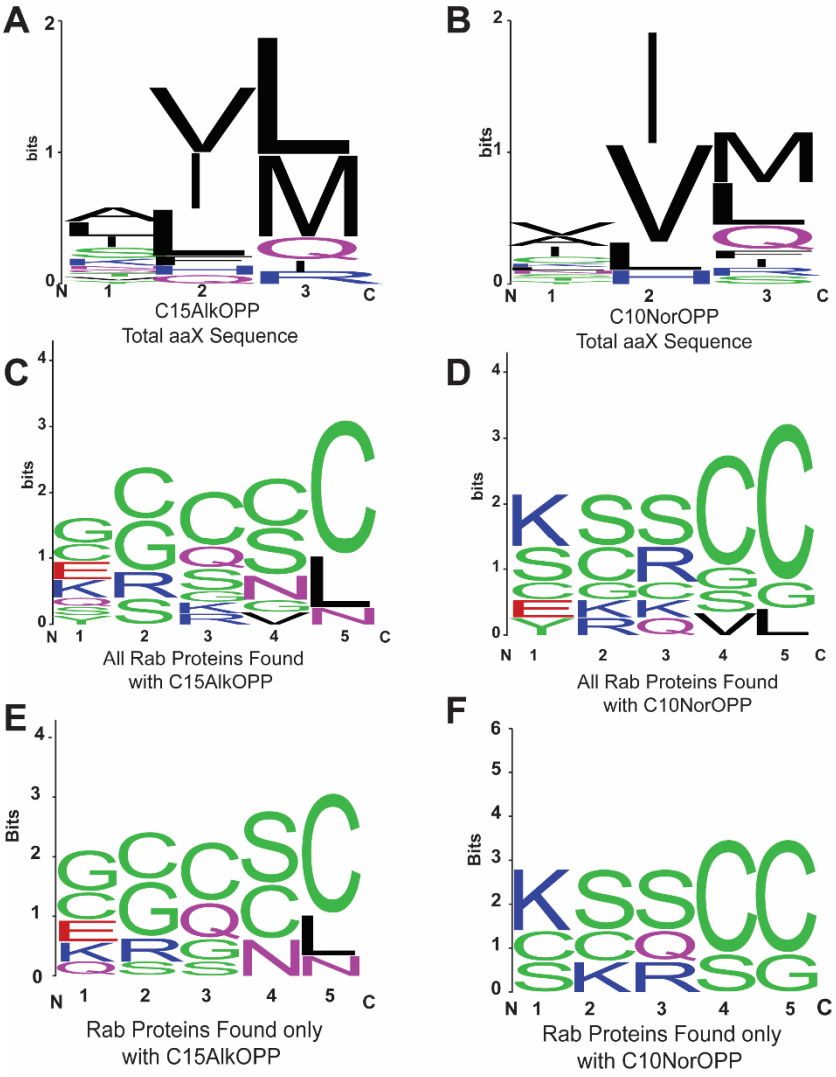


Figure S6: Sequence Logos examining prenylation sites of proteins found in proteomic experiments.

Sequence logos exploring the differences in the prenylation site of proteins labeled with C15AlkOPP or C10NorOPP. FTase and GGTase I substrates were analyzed separately from GGTase II substrates (Rab proteins). Sequence logos were generated with the (https://weblogo.berkeley.edu/logo.cgi) tool. (A) Comparison of the "aaX" residues in all proteins found with C15AlkOPP (data ungrouped). Enzymatically modified cysteine was not included in the "CaaX" analysis. (B) Comparison of the "aaX" residues in all proteins found with C10NorOPP (data ungrouped). (C-D). Comparison of the 5 pre-C-terminal residues of all the Rab Proteins, both commonly found between the two probes and exclusively found with (C) C15AlkOPP, (D) C10NorOPP. (E-F) Comparison of the 5 most C-terminal residues of the Rab Proteins found with only (E) C15AlkOPP of (F) C10NorOPP.

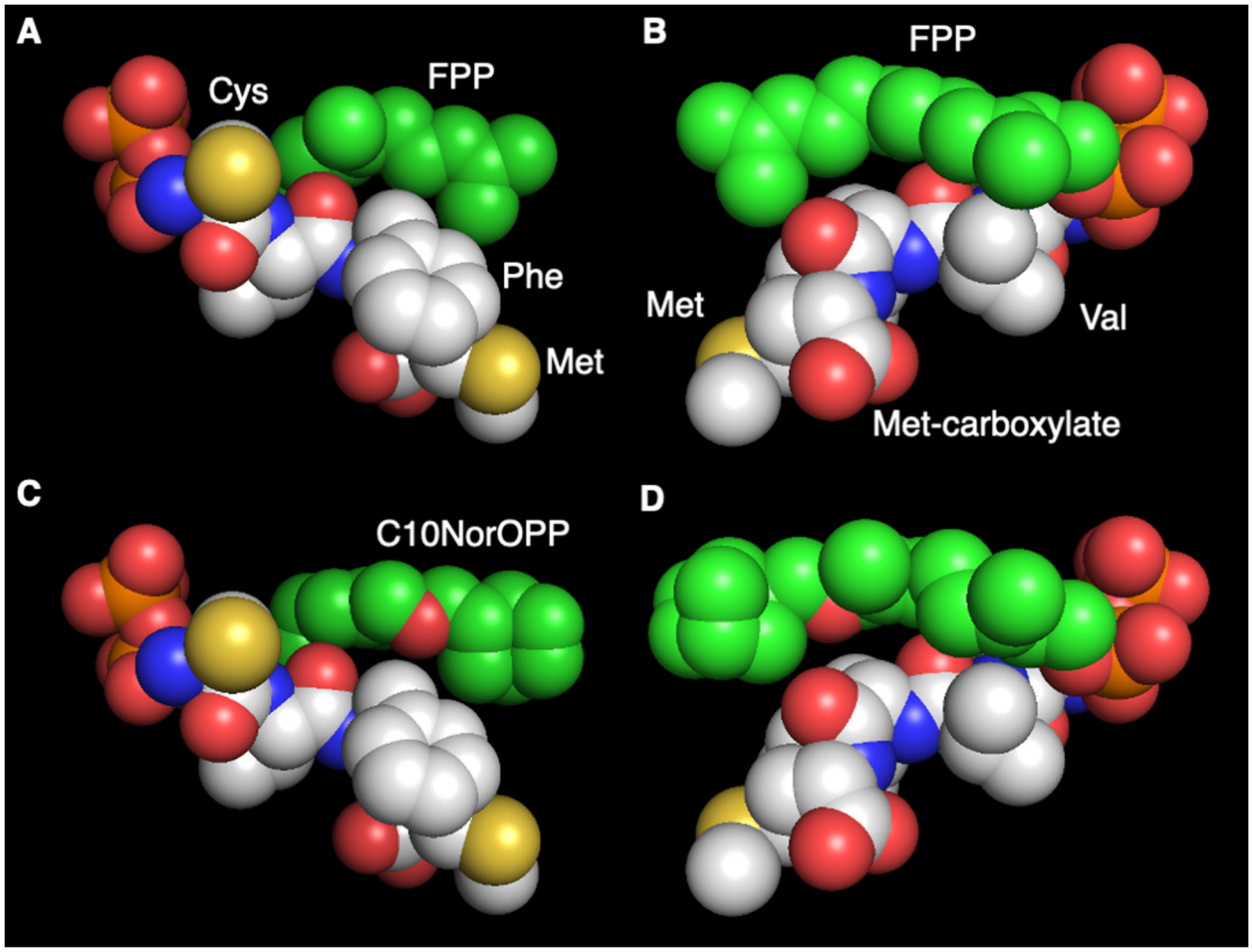


Figure S7. Comparison of peptide-isoprenoid interactions in the FTase ternary complex.
The structure of the FPP-containing ternary complex is from pdb file 1JCR. The structure of the C10NorOPP complex was obtained from docking. In all views, the isoprenoid along with the peptide substrate CVFM is shown. (**A**) Front view and (**B**) rear view of FPP complex showing interactions between FPP and Phe residue in the front and Val in the rear. (**C**) Front view and (**D**) rear view of C10NorOPP complex showing interactions between C10NorOPP and Phe residue in the front and Val in the rear. Color scheme: C (white in peptide, green in isoprenoid); N (blue); O (red); P (orange); S (yellow).

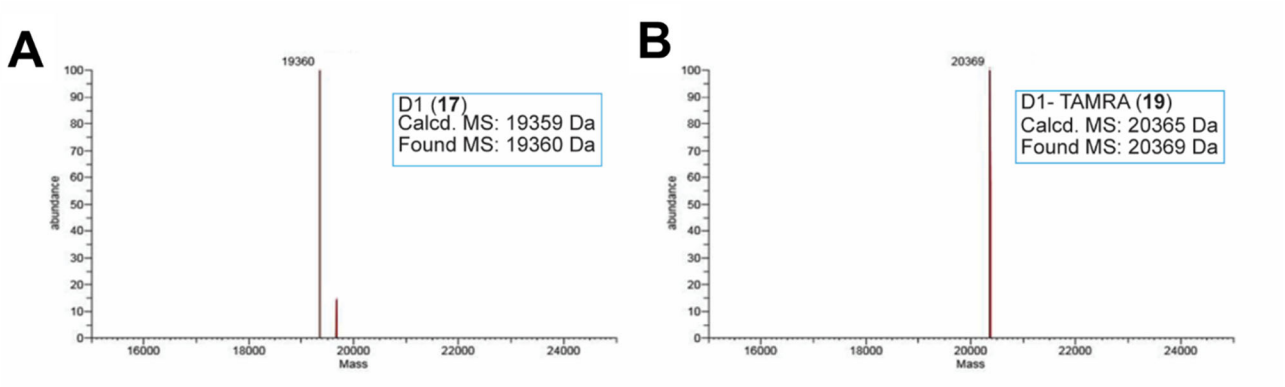


Figure S8: LC-MS analysis of DARPin D1 protein (17) and D1-TAMRA conjugate (19).
(A) A solution of D1 protein (**17**, 18 µL of 5 µM in PBS) was analyzed by LC-MS. (B) A solution of D1-TAMRA protein (**19**, 18 µL of 5 µM in PBS), obtained by enzymatic prenylation with C10NorOPP followed by reaction with **19** was analyzed by LC-MS.

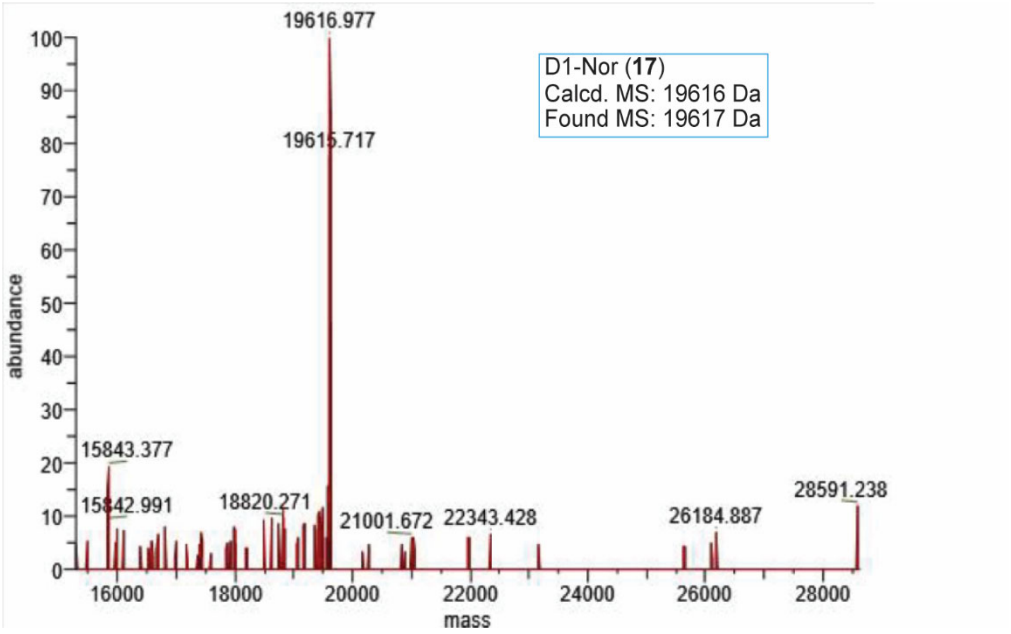


Figure S9: LC-MS characterization of D1-Nor (17).
A solution of D1-Nor protein (**17**, 18 µL of 5 µM in PBS), obtained by enzymatic prenylation of D1 (**16**) with C10NorOPP (**1**) was analyzed by LC-MS.

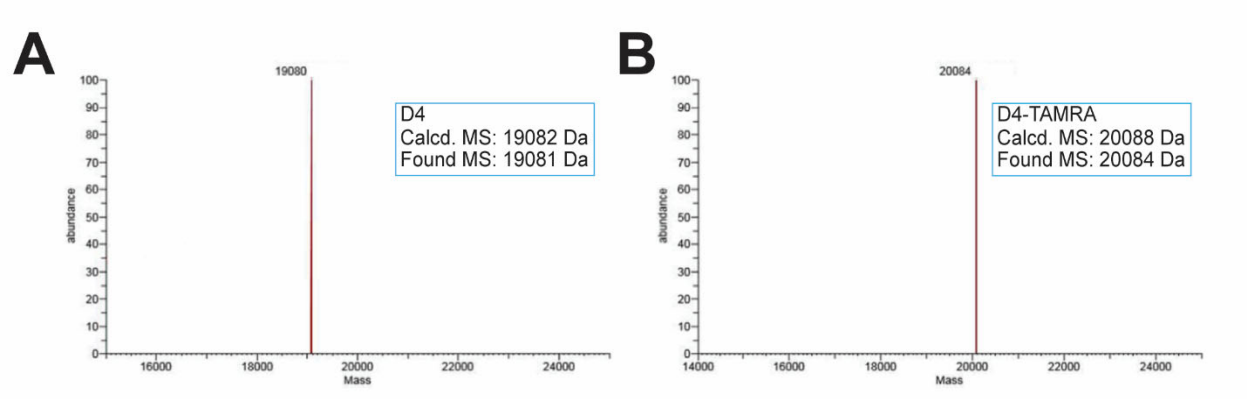


Figure S10: LC-MS characterization of non-binding control DARPin D4 protein and D4-TAMRA conjugates.
(A) A solution of D4 protein (18 µL of 5 µM in PBS) was analyzed by LC-MS. (B) A solution of D4-TAMRA protein (18 µL of 5 µM in PBS) obtained by enzymatic prenylation with C10NorOPP followed by reaction with D4 was analyzed by LC-MS

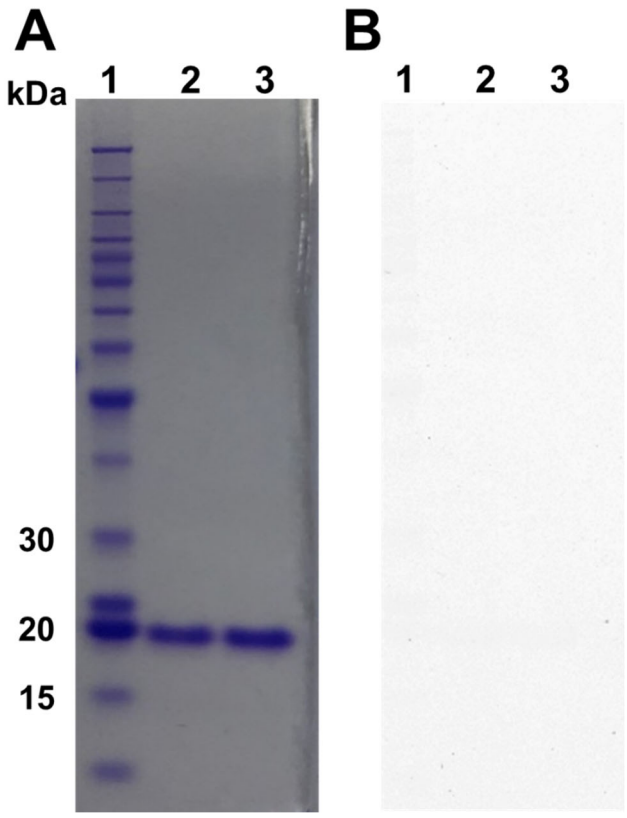


Figure S11: In-gel fluorescence assay of D1 (17) treated with excess TAMRA-Tetrazine (13a).
D1 (**17**, 200 µL,10 µM) was reacted with 10-fold excess of TAMRA-Tetrazine (**13**) at rt for overnight. The protein samples were denatured at 95 ºC for 5 min in SDS PAGE loading buffer containing DTT (0.5 mM) and then fractionated on a NuPAGE Bis-Tris protein gel (Thermo Fisher Scientific, Cat: NP0321PK2) via electrophoresis at 120 V. The gel was scanned with a fluorescent gel scanner (Typhoon FLA 9500 (GE Healthcare)) for detection of TAMRA fluorescence gel and stained with Coomassie blue. No fluorescent labeling was observed, indicating that the norbornene is required for labeling and that no detectable nonspecific labeling occurs. Lane 1: Ladder; Lane 2: D1; Lane 3: D1 reacted with TAMRA. (A) Gel after Coomassie blue staining. (B) Gel after Fluorescence scanning.

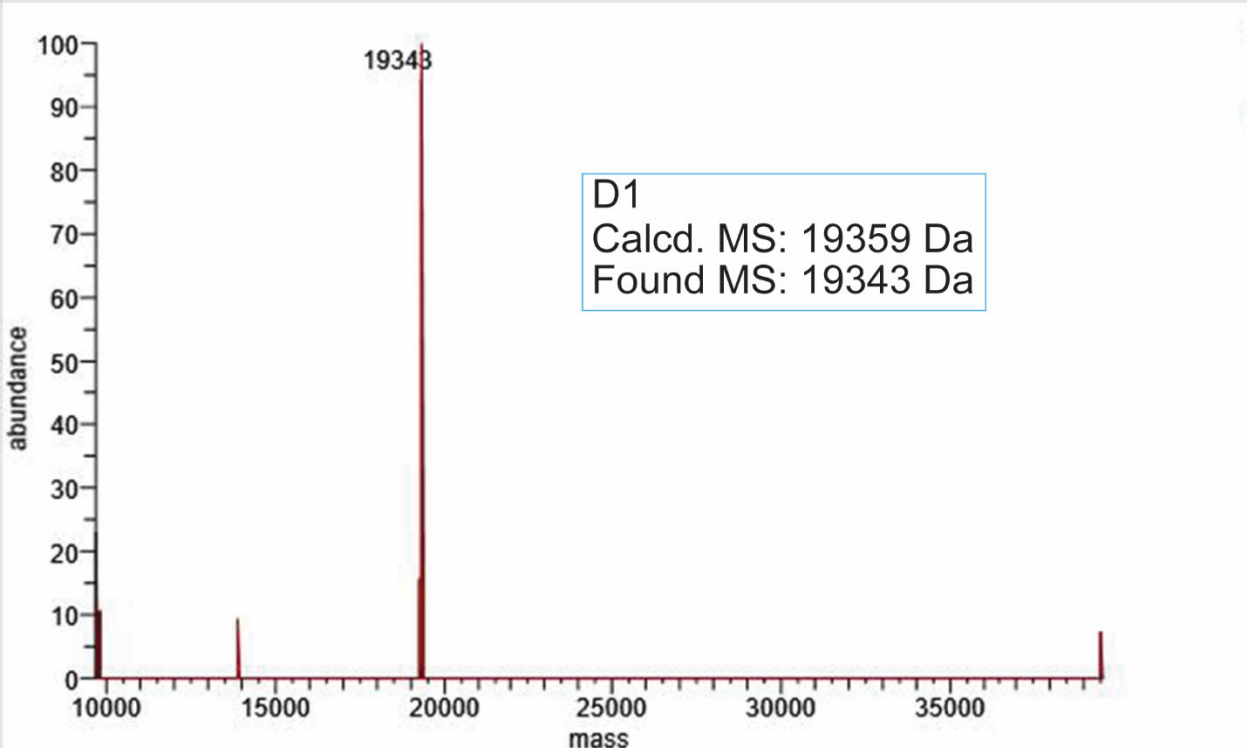


Figure S12: LC-MS of D1 (17) after reaction with Tetrazine-TAMRA (13).
D1 (**17)**, 200 µL, 10 µM) was reacted with 10-fold excess of TAMRA-Tetrazine (**13b**) at rt overnight. For LC-MS, the protein sample was exchanged into ultrapure water with an Amicon Ultra-0.5 centrifugal filter (10 kDa cutoff, Millipore) and then analyzed using an Orbitrap Elite Hybrid Mass Spectrometer.

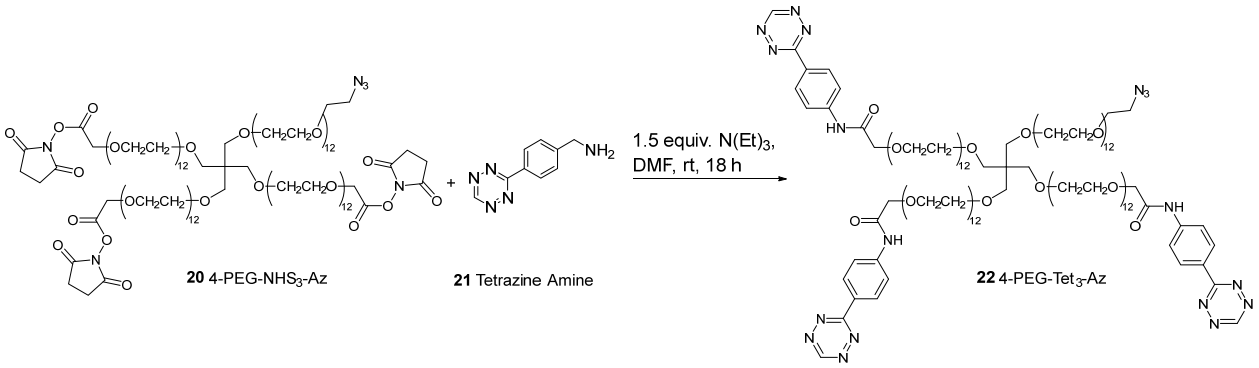


Figure S13: Synthesis of 4-PEG Tet$_3$-Az (22) from 4-PEG-NHS$_3$-Az (20).

A

4-PEG-NHS_3-Az	Tetrazine amine	Triethylamine
10 mg	2.8 mg (3 equiv.)	2.3 mg (4.5 equiv.)
1 mg	0.37 mg (4 equiv.)	0.3 mg (6 equiv.)
1 mg	0.56 mg (6 equiv.)	0.45 mg (9 equiv.)
1 mg	0.9 mg (10 equiv.)	0.76 mg (15 equiv.)

B

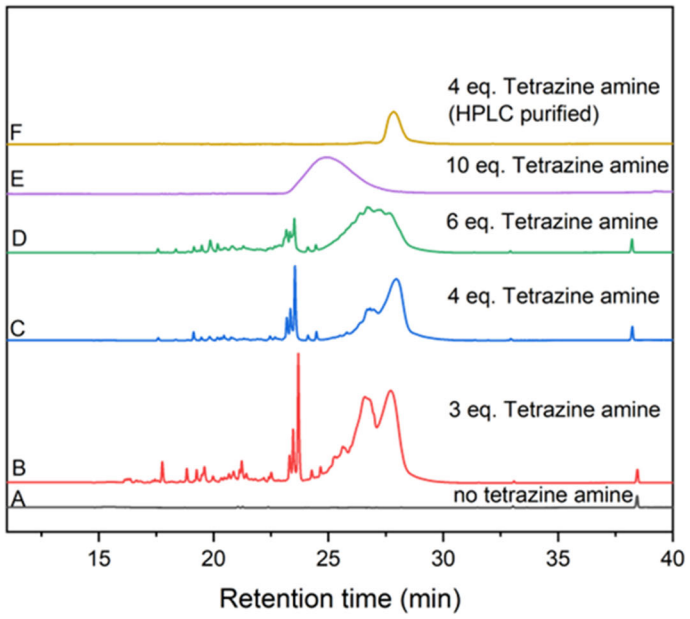


Figure S14: Optimization of the synthesis of 4-PEG -Tet_3-Az (22).
(A) Table summarizing the different reaction conditions. (B) Crude reaction mixtures were analyzed via reverse-phase HPLC, with detection at 280 nm. The trace A: 4-PEG-NHS_3-Az; trace B: crude reaction with 3 equiv. tetrazine amine and 4.5 equiv. triethylamine; trace C: crude reaction with 4 equiv. tetrazine amine and 6 equiv. triethylamine; trace D: crude reaction with 6 equiv. tetrazine amine and 9 equiv. triethylamine; trace E: crude 10 equiv. tetrazine amine and 15 equiv. triethylamine; trace F: After HPLC purification of the peak from 27 min to 30 min retention time in the reaction containing 4 equiv. of tetrazine amine. The desired 4-PEG-Tet_3-Az **(22)** had the longest retention time compared to possible di- and mono-tetrazine species, with a shift towards longer retention times observed for hydrophobic tetrazine addition. The addition of large excess amounts of tetrazine amine led to decreased yield of the desired product as seen in trace E.

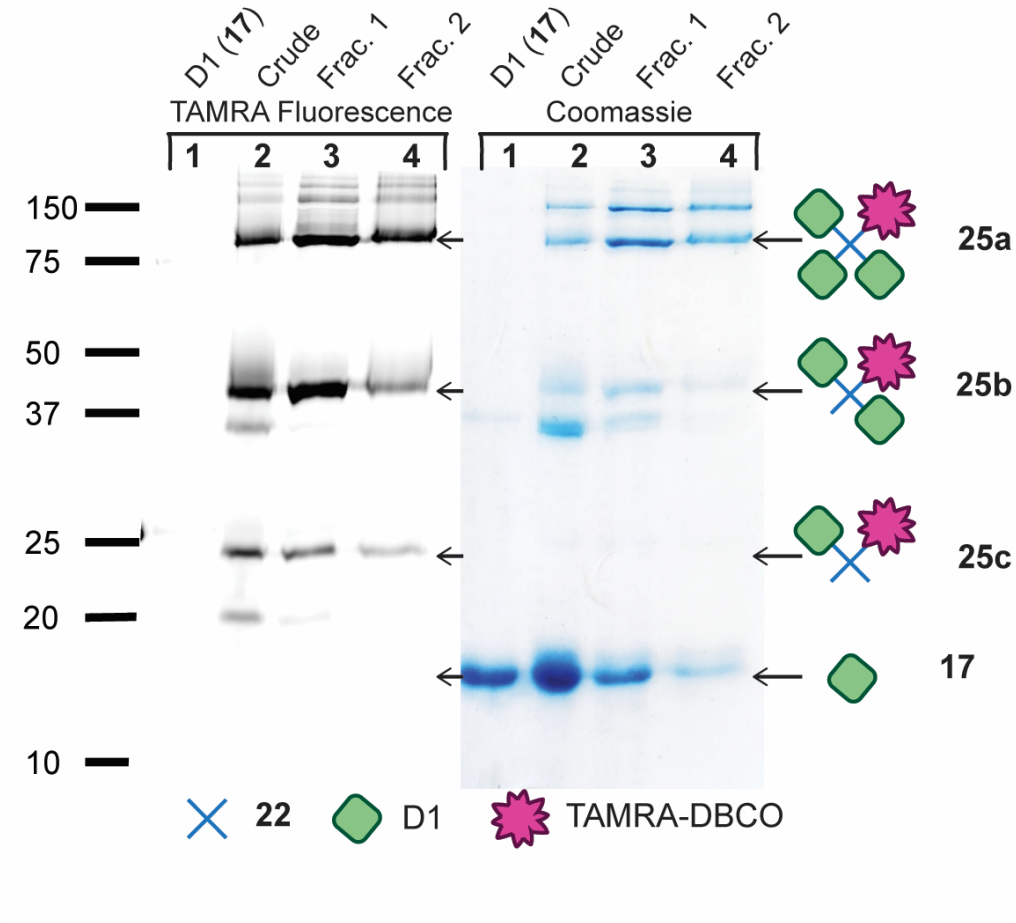


Figure S15: Examination of Purity of Trimeric DARPin (25a) with in-gel fluorescence.

SDS-PAGE analysis of purification of the 4-PEG-D1$_3$-TAMRA (**25a**) conjugate. Left panel: Fluorescence scan to visualize TAMRA fluorescence; Right panel: Coomassie stain to visualize protein. Lane 1: Unmodified D1; Lane 2: Crude reaction mixture post IEDDA ligation and SPAAC reaction. Lane 3: Fraction 1 from Ni-NTA purification containing 4-PEG-D1$_3$-TAMRA (**25a**) along with some dimeric and monomeric species; Lane 4: Fraction 2 from Ni-NTA purification containing mostly 4-PEG-D1$_3$-TAMRA (**25a**) and lesser amounts of dimeric and monomeric species.

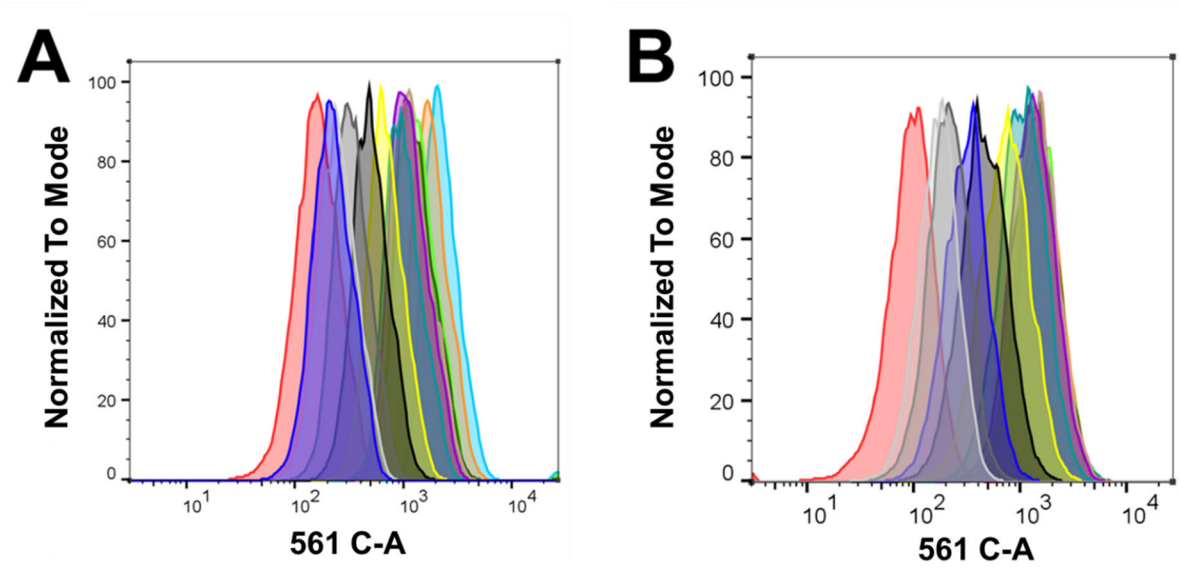


Figure S16: Representative flow cytometry on EpCAM$^+$ MCF-7 cells treated with DARPin constructs.
MCF7 cells were incubated with the following 10 concentrations of 4-PEG-D1$_3$-TAMRA (**25a**) or D1-TAMRA **(19)**: 125 nM, 62.5 nM, 31.2 nM, 15.6 nM, 7.8 nM, 3.9 nM, 1.9 nM, 0.9 nM, 0.4 nM, and 0.2 nM which were serially diluted in PBSA in the dark and left for 45 min at 4^{o}C. After incubation, the cells were washed with PBSA (2 x 1 mL) before analysis. Affinity titration measurements were performed using a BD Fortessa X-20 flow cytometer at the University Flow Cytometry Resource (UFCR) and 1x10^4 cells were counted. The TAMRA fluorescence was recorded using the yellow-green 561 nm excitation laser, 50 mW equipped with 585/15 band pass filter. The data was recorded with the help of FACS Diva software. Data analysis was performed with FlowJo software (v10.8.1). (A) Flow cytometry data obtained using 4-PEG-D1$_3$-TAMRA (**25a**). (B) Flow cytometry data obtained using D1-TAMRA (**19**).

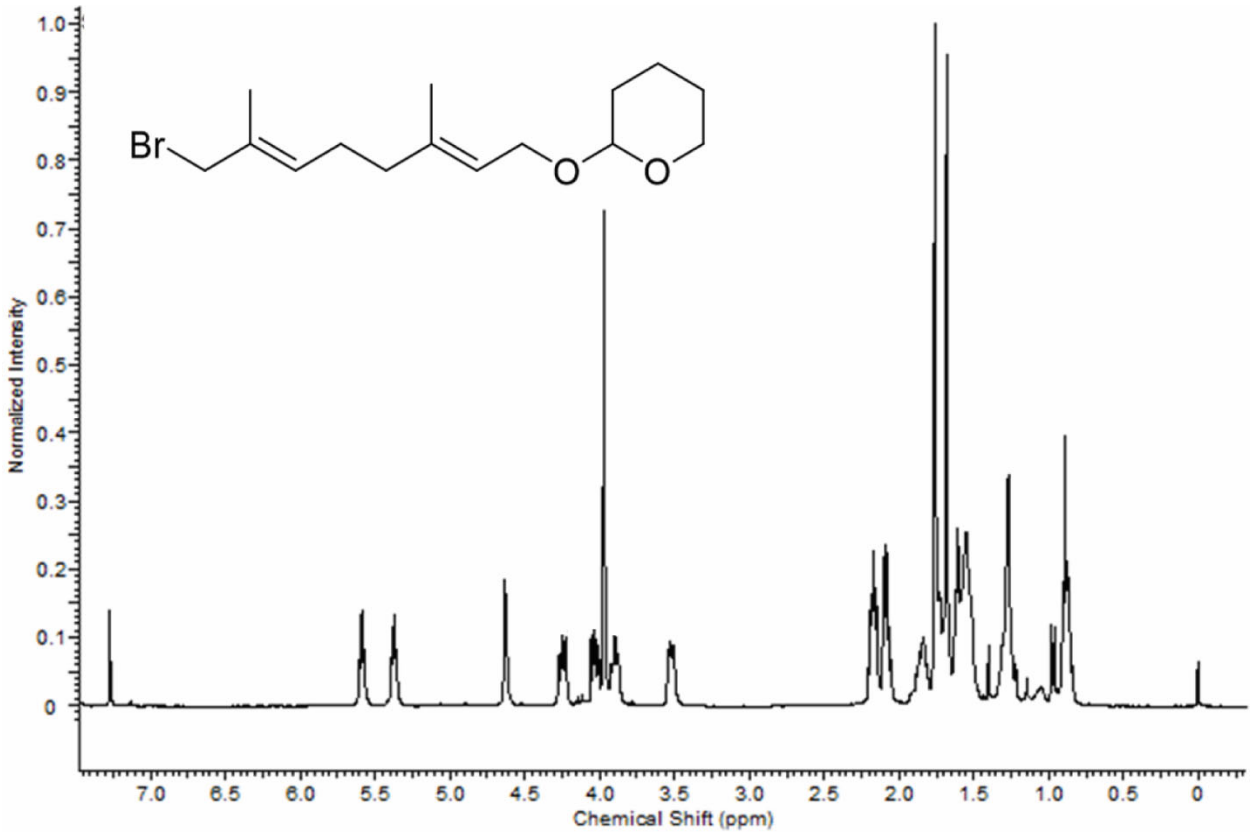


Figure S17: ^{1}H NMR of 5.

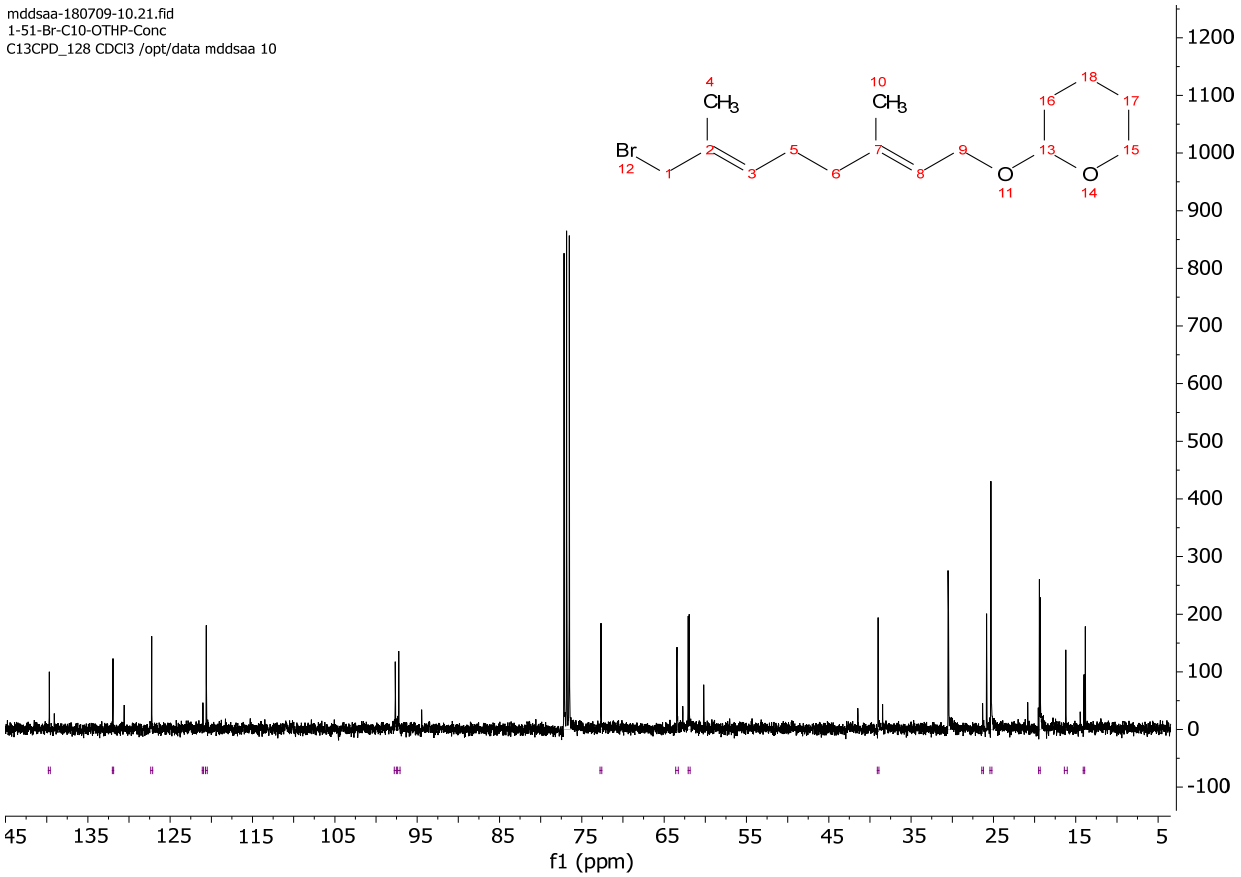


Figure S18: ^{13}C NMR of 5.

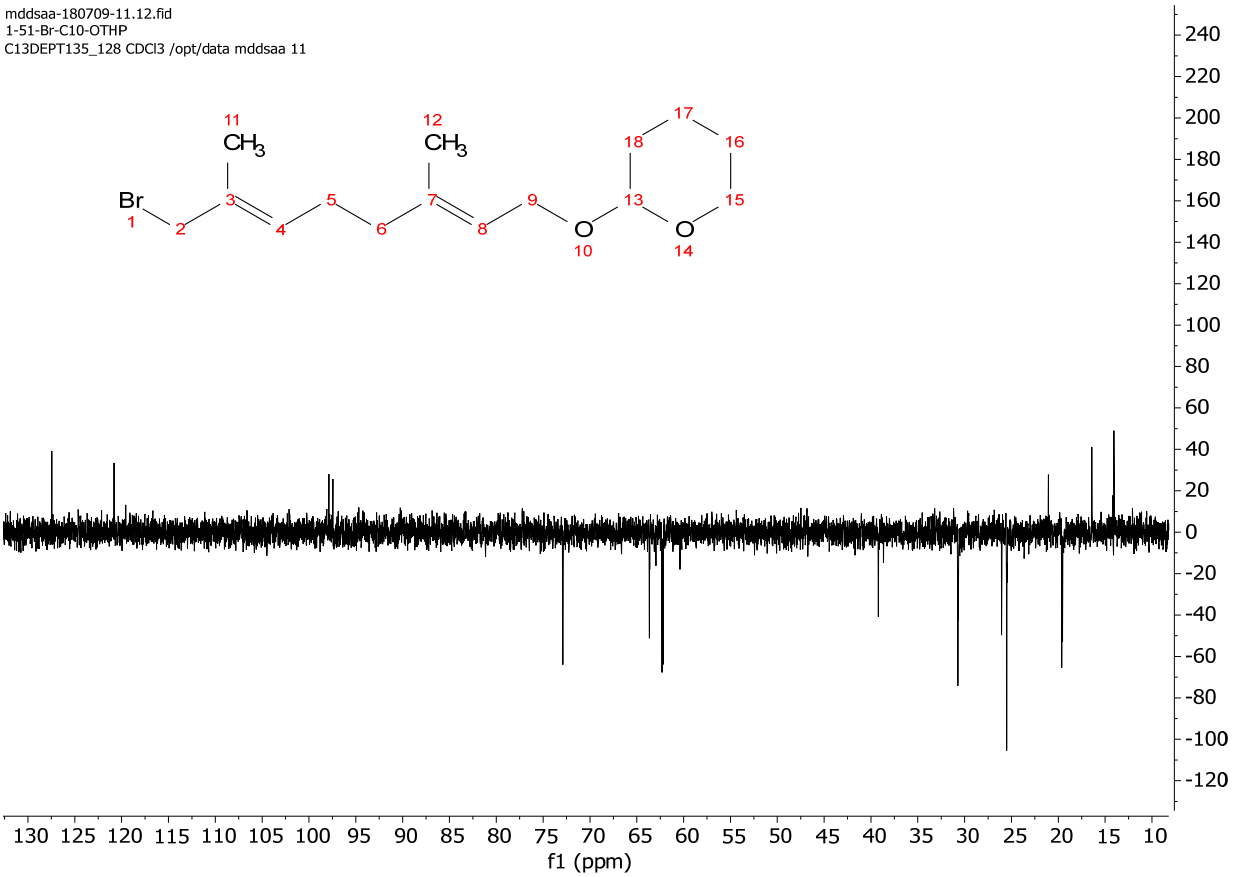
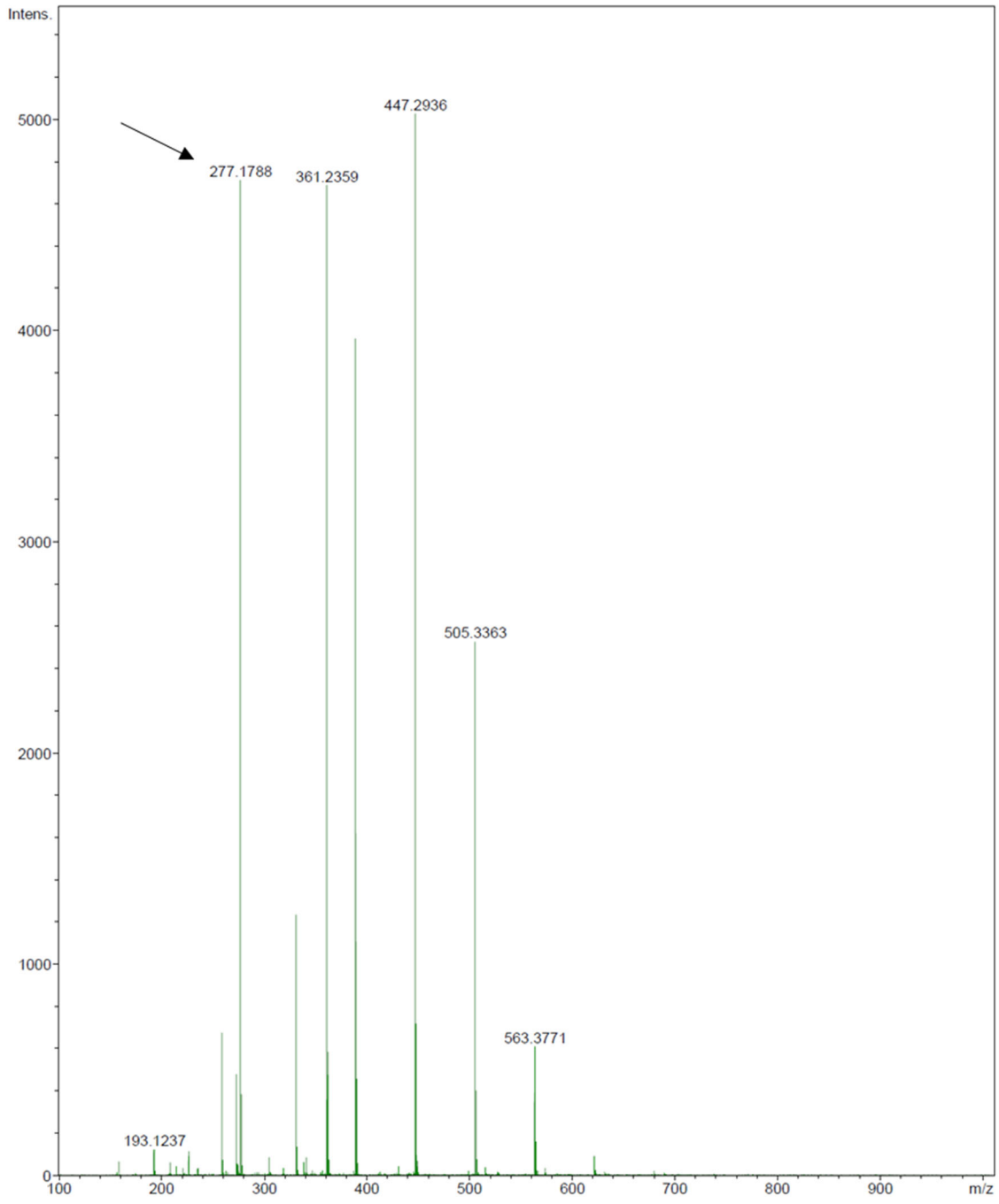


Figure S19: ^{13}C DEPT NMR of 5.
Note: sp^3 and sp hybridized carbons are positive and sp^2 hybridized carbons are negative.



FigureS20: HRMS of 5.
Spectrum for [$NaC_{15}H_{26}O_3$], the major ion formed after loss of bromine. PPG 425 as internal standard. Black arrow indicates sample peak.

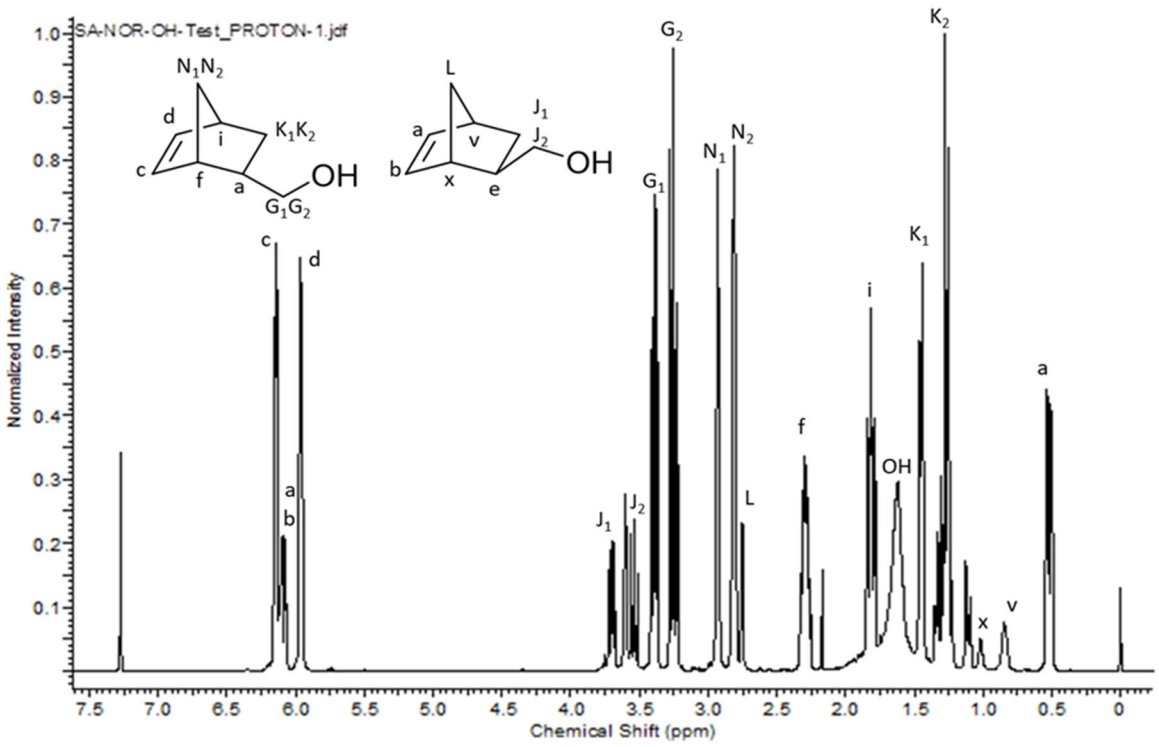


Figure S21: ^{1}H NMR of 6 (a mixture of 6a and 6b).
^{1}H NMR (400 MHz, $CDCl_3$) δ ppm; 6.14 (*endo,* dd, *J* = 5.8, 3.1, 1H), 6.09 (*exo*, dd, *J* = 9.0, 3.0, 0.4 H),5.96 (*endo*, dd, *J* = 5.8, 2.9, 1H), 3.70 (*exo*, dd, *J* = 10.5, 6.4, 0.2 H), 3.54 (*exo,* dd, *J* = 10.5, 8.8, 0.2 H), 2.75 (d, *J* = 2.6, 1H), 3.40 (*endo*, dd, *J* = 10.5, 6.5, 1H), 3.25 (*endo*, dd, *J* = 10.4, 8.9, 1H), 2.93 (*endo*, s, 1H), 2.81 (*endo/exo*, d, *J* = 4.1, 1.2 H), 2.29 (*endo/exo* dt, *J* = 9.1, 3.3, 1.2 H), 1.82 (*endo/exo,* ddd, *J* = 11.6, 9.1, 3.9, 1.2H),1.61 (ddt, *J* = 6.8, 4.3, 2.1, 1H), 1.47 (s, 1H), 1.47 – 1.44 (m, 7H), 1.38 – 1.20 (m, 4H), 1.11 (dt, *J* = 11.6, 3.9, 1H), 0.52 (ddd, *J* = 11.6, 4.5, 2.6, 1H).

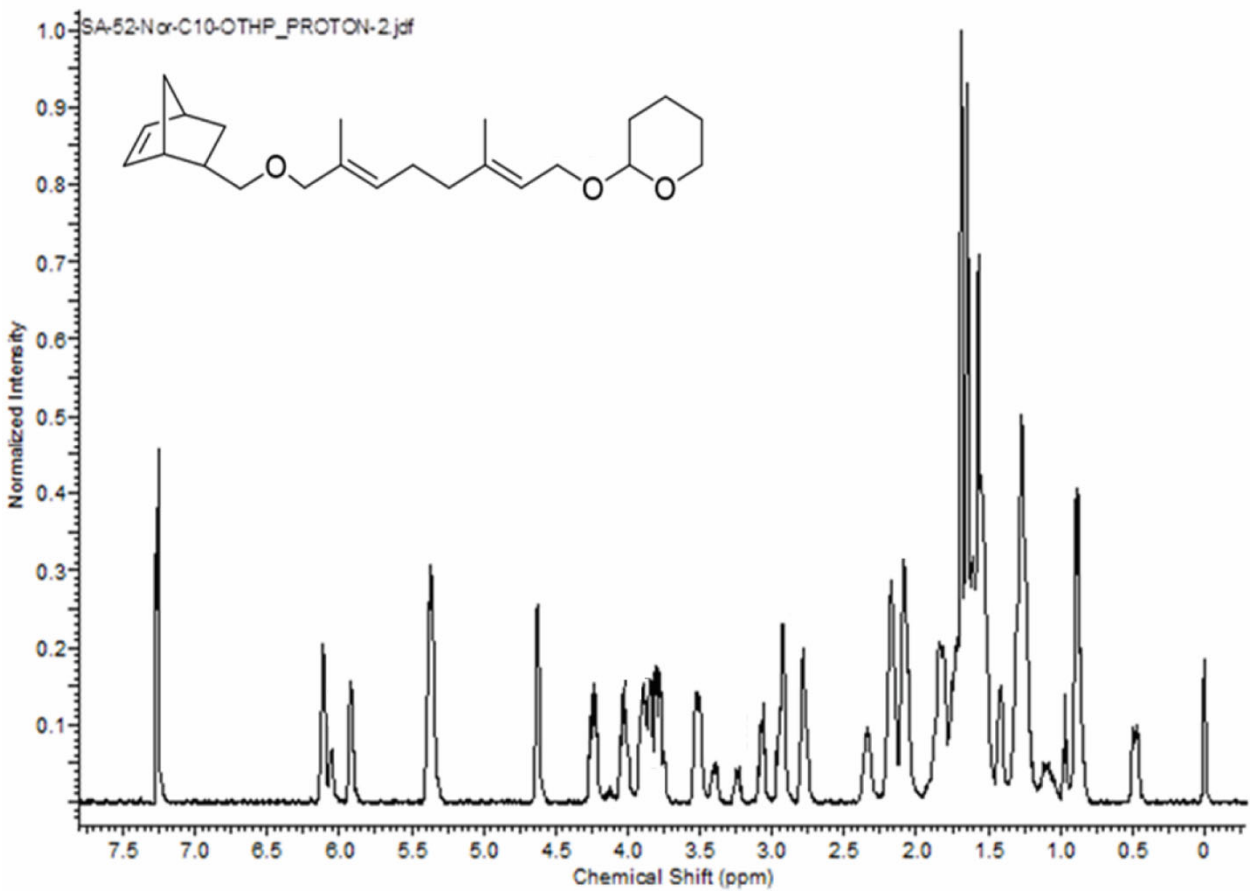


Figure S22: ^{1}H NMR of 7 (a mixture of 7a and 7b).

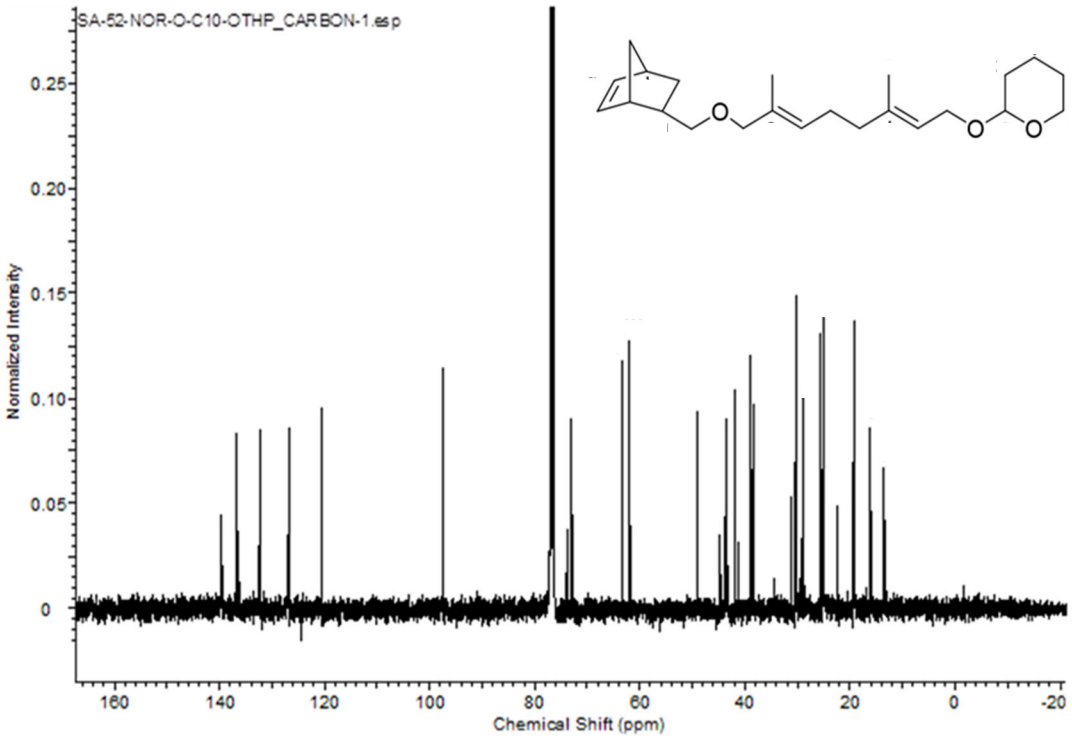


Figure S23: ^{13}C NMR of 7 (a mixture of 7a and 7b).

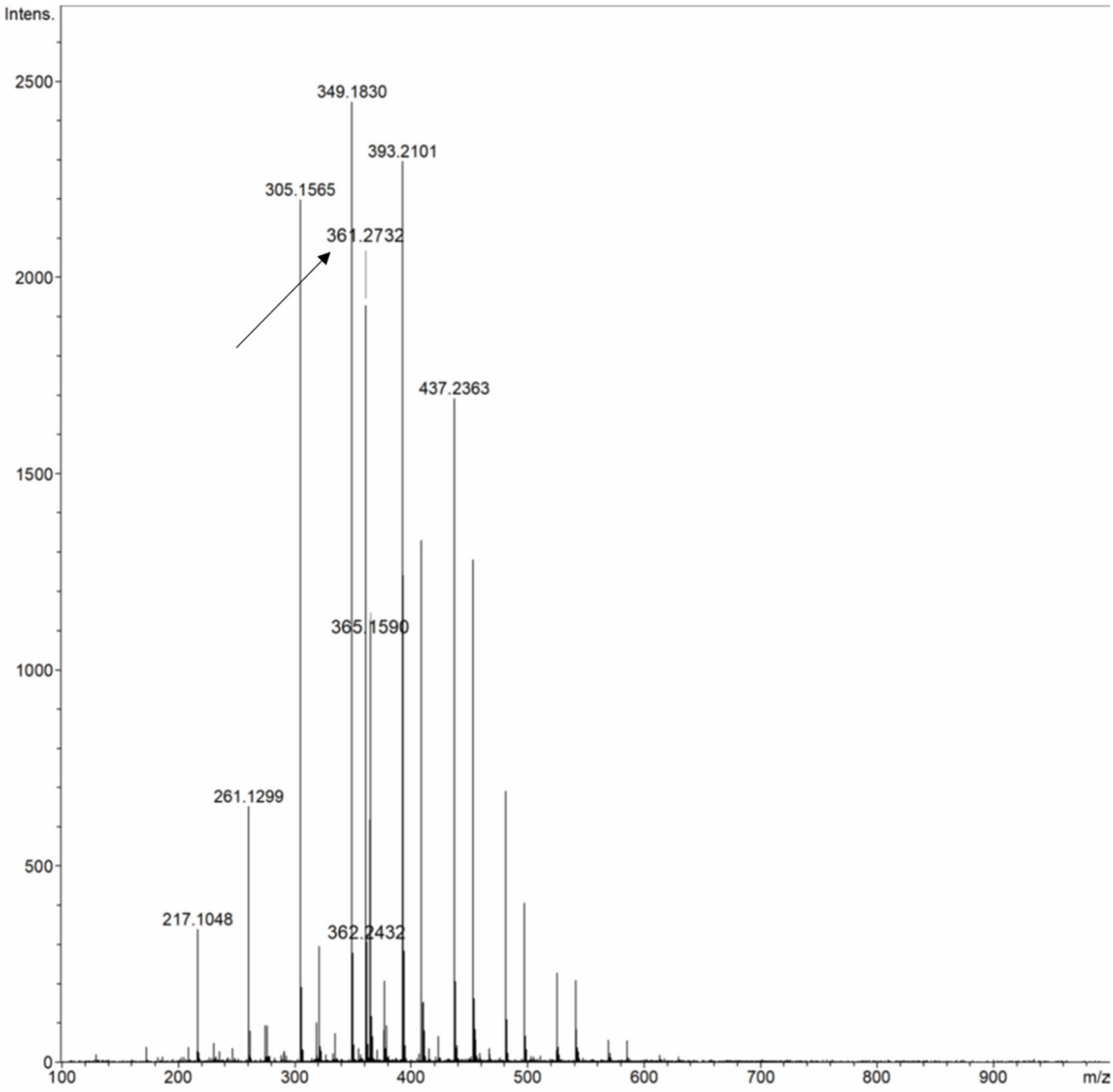


Figure S24: HR-MS of 7 (a mixture of 7a and 7b) with PEG 400 internal standard. Sample peak indicated with black arrow.

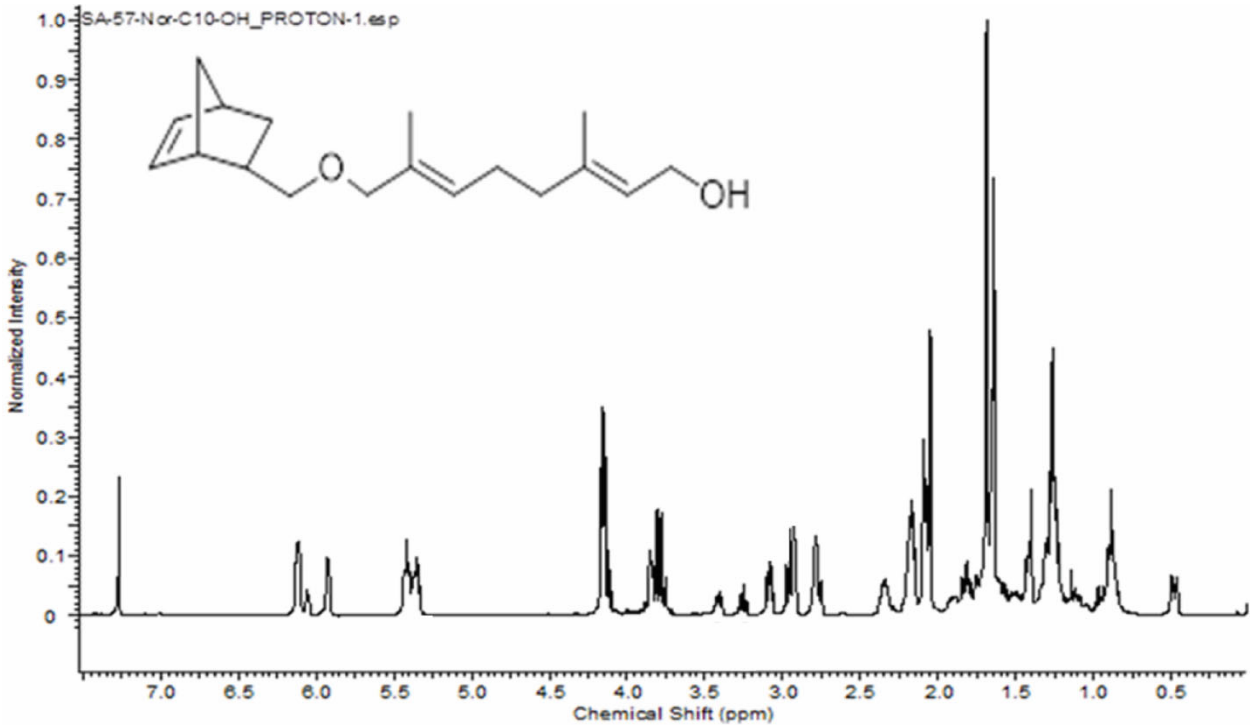


Figure S25: ^{1}H NMR of 8 (a mixture of 8a and 8b).

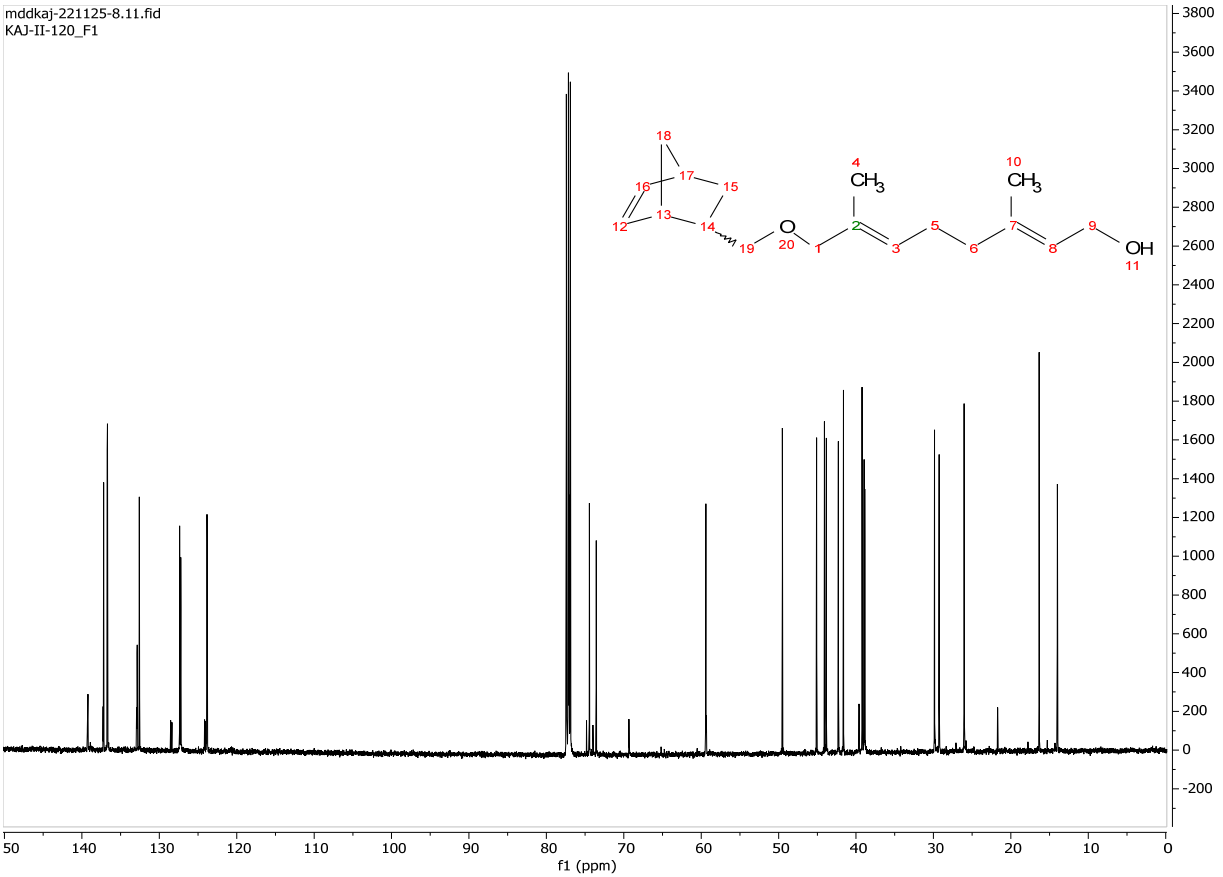


Figure S26: ^{13}C NMR of 8 (a mixture of 8a and 8b).
Compound **8** was resynthesized after all experiments were completed using a commercial **6** that was a 50/50 mixture of endo/exo.

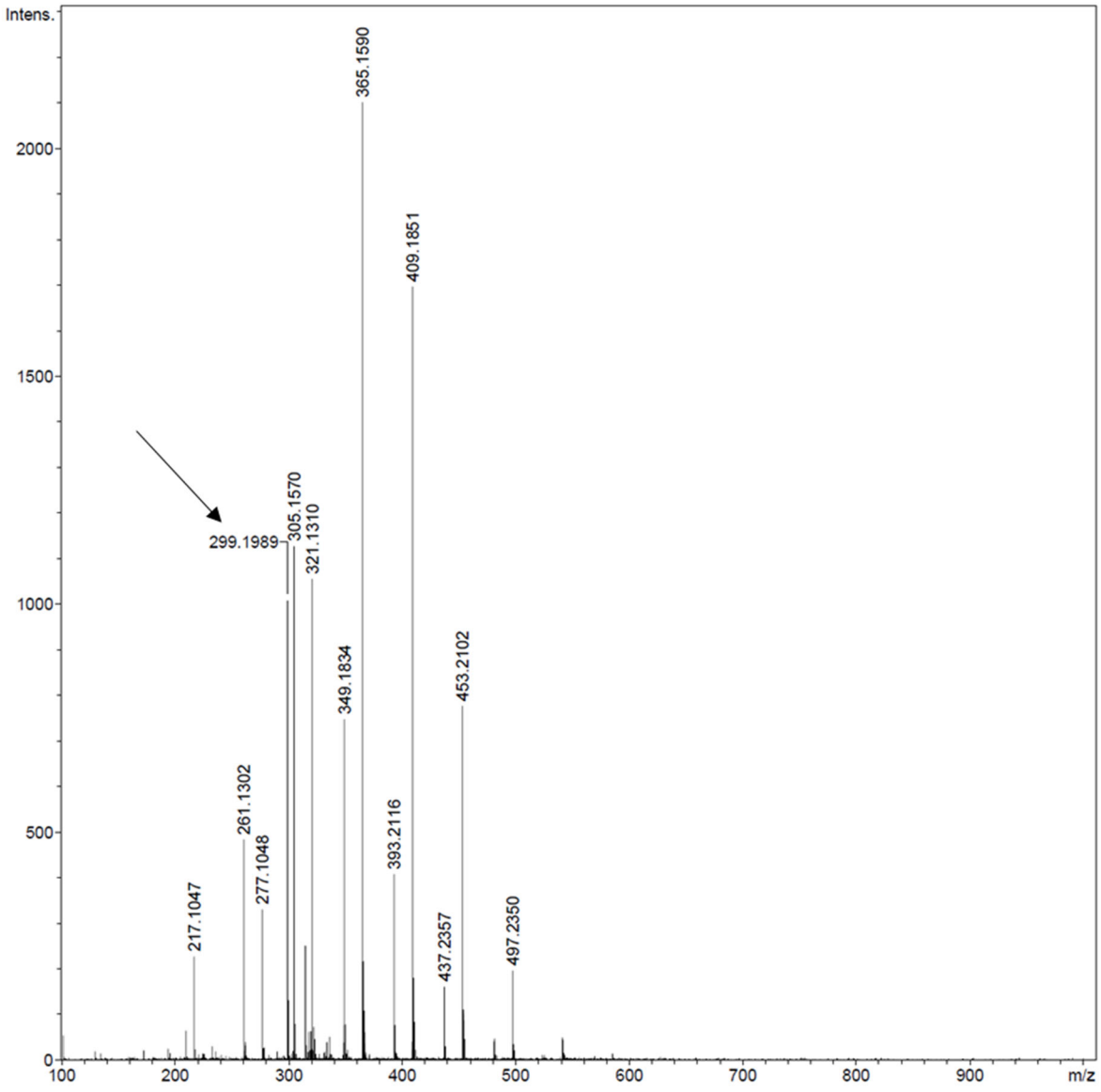


Figure S27: HRMS of 8 (a mixture of 8a and 8b) with PEG 400 internal standard. Sample peak indicated with black arrow.

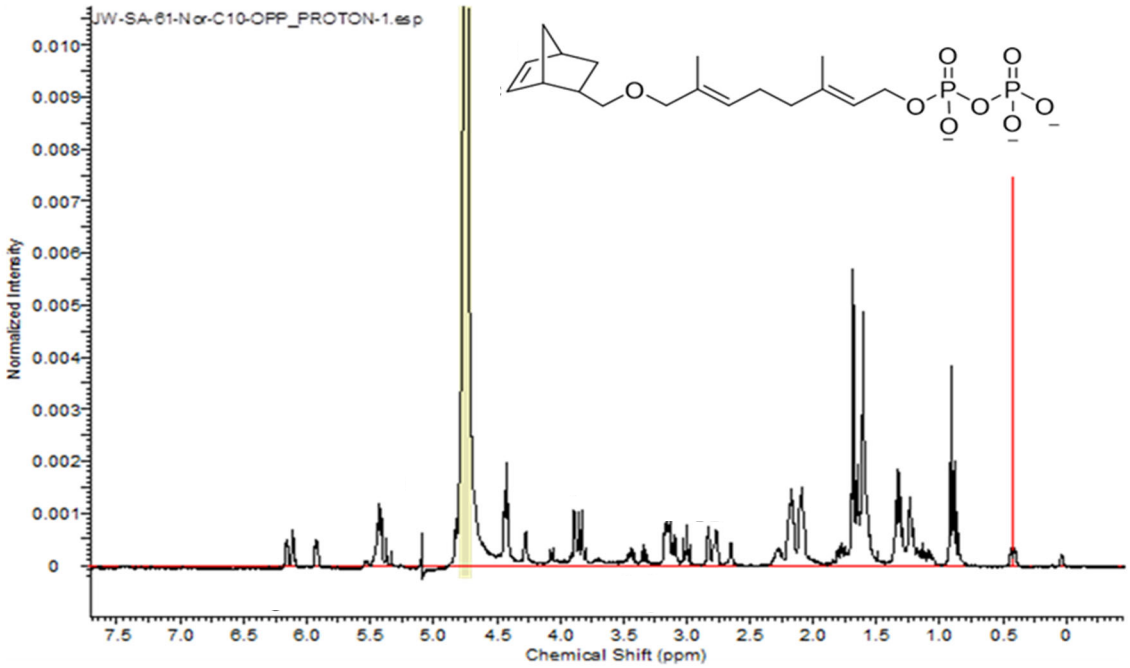


Figure S28: ^{1}H NMR of C10NorOPP (1, a mixture of 1a and 1b).

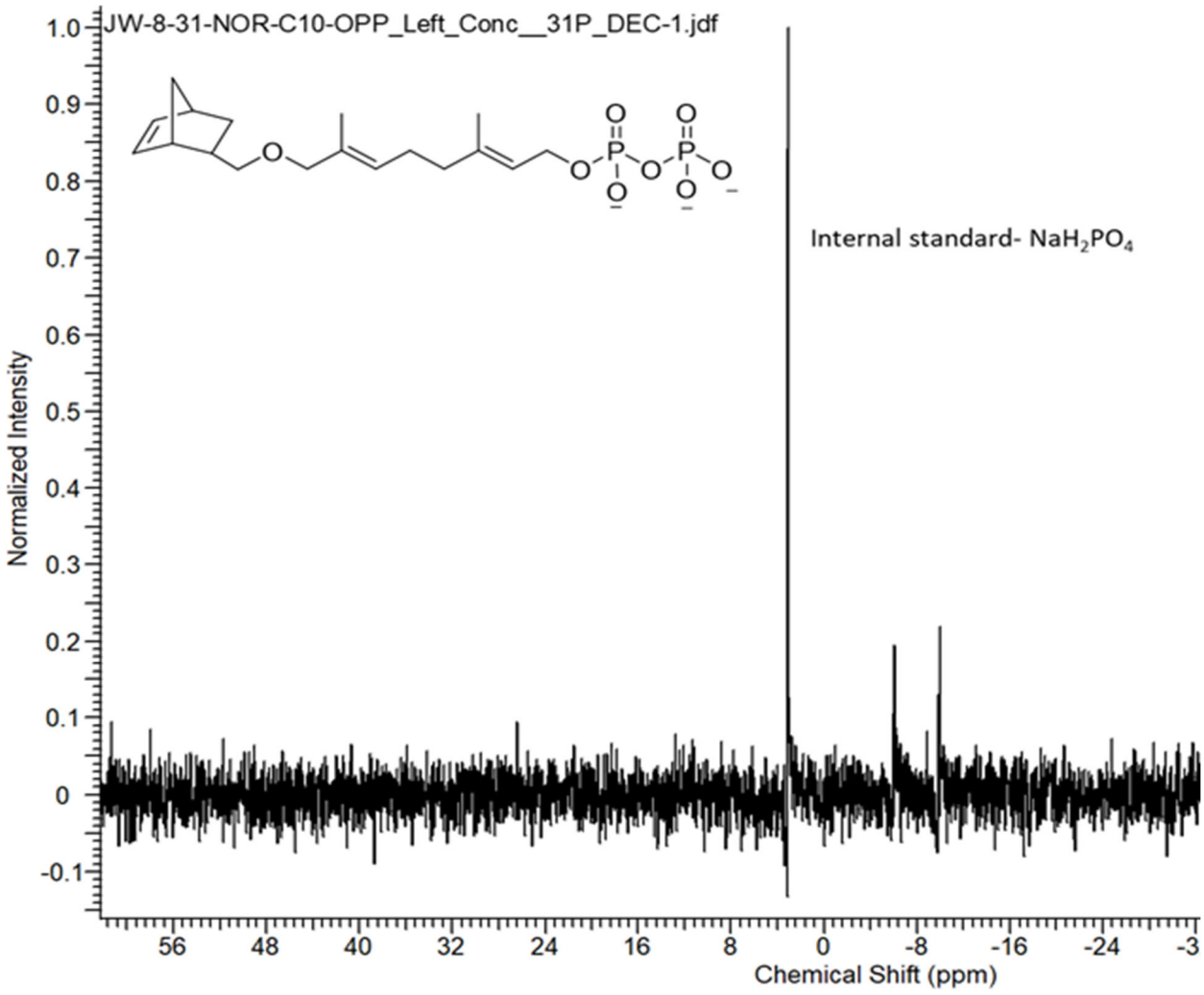


Figure S29: ^{31}P NMR of C10NorOPP (1, a mixture of 1a and 1b) including NaH_2PO_4 as an internal standard.

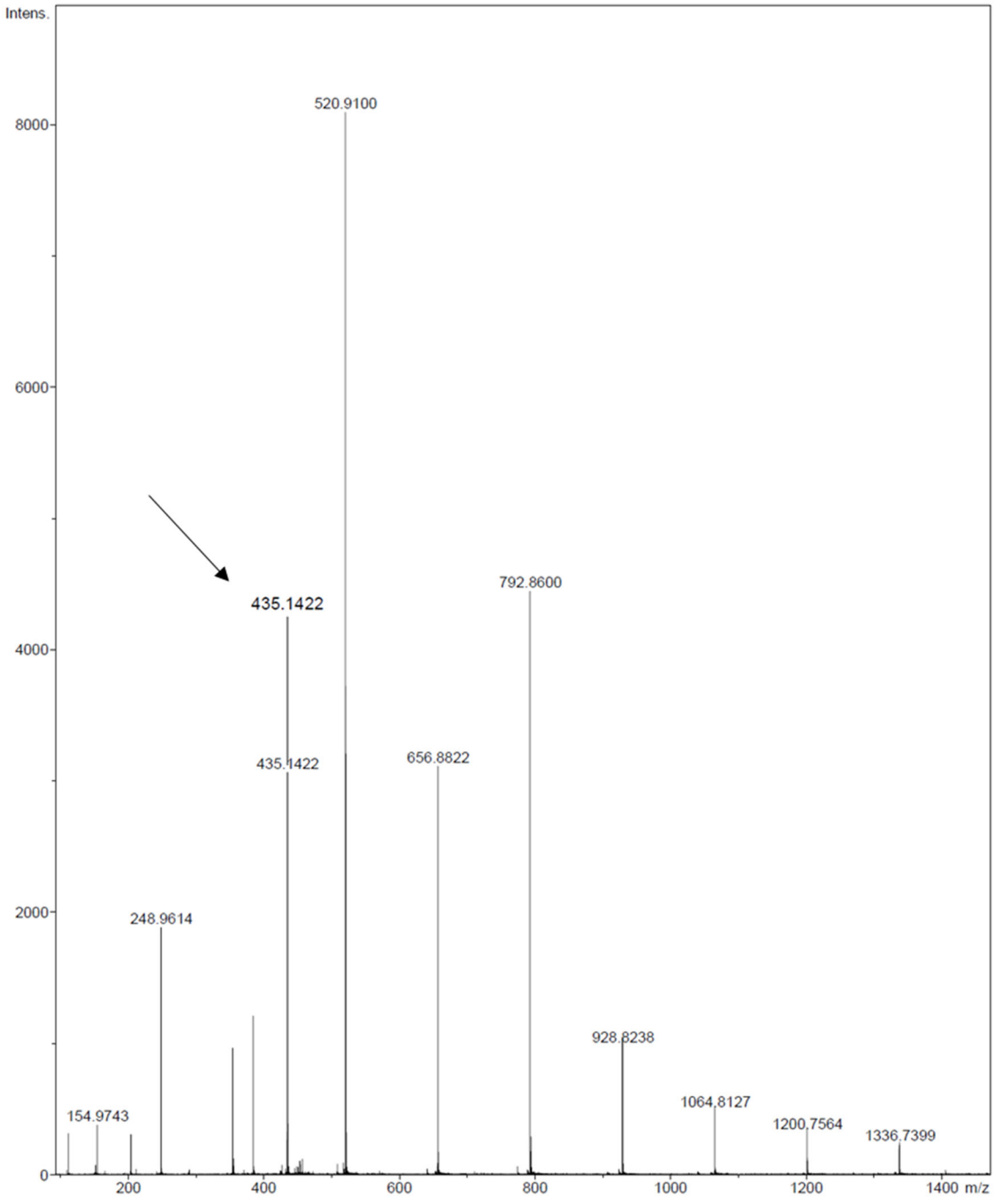


Figure S30: HRMS of C10NorOPP (1, a mixture of 1a and 1b).

References

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