A conformationally-restricted aza-BODIPY platform for stimulus-responsive probes with enhanced photoacoustic properties

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Materials. Copper(II) chloride dihydrate and triethylamine were purchased from Acros Organics. Allyl bromide and cyclohexane were purchased from Alfa Aesar. 4-Methoxyphenylboronic acid was purchased from Ark Pharm. NADPH tetrasodium salt was purchased from CalBioChem. Deuterated solvents were purchased from Cambridge Isotope Laboratories. 6-Hydroxy-1-tetralone was purchased from Combi-Blocks. 1×PBS (without calcium or magnesium) was purchased from Corning. Ammonium chloride, dichloromethane, dichloroethane, diethyl ether, ethyl acetate, ethylene glycol, glacial acetic acid, hexanes, tetrahydrofuran, and potassium phosphate monobasic were purchased from Fisher Scientific. Agarose LE was purchased from Gold BioChem. TLC plates, and silica and alumina for flash chromatography, were purchased from Machery-Nagel. Chloroform, methanol (anhydrous) and sodium hydroxide were purchased from Macron Fine Chemicals. Ethyl isocyanoacetate was purchased from Matrix Scientific. Fluorinated ethylene propylene (FEP) tubing (wall thickness 0.01”, inner diameters 0.08” and 0.12”) was purchased

from McMaster-Carr. 1-Tetralone, 2,6-dichlorophenol, 2-bromo-2-methylpropanamide, 4'-aminoacetophenone, 4'-methoxyacetophenone, 6-methoxy-1-tetralone, acetic anhydride, celite, di-*tert*-butyl-dicarbonate, bromoethane, ethyl iodide, methyl iodide, *N*-iodosuccinimide, potassium carbonate, potassium hydroxide, p-toluenesulfonic acid hydrate, sodium azide, sodium bicarbonate, sodium borohydride, sodium nitrite, sodium sulfate, sodium thiosulfate, TEMPO, tetrakis(triphenylphosphine)palladium(0), and triflic acid were purchased from Oakwood Chemical. 1,4-Dioxane, acetyl chloride, boron trifluoride diethyl etherate, bromine, DBU, *N,N*-dimethylacetamide, rat liver microsomes, sodium hydride, styrene, *tert*-butyl nitrite, and all additional anhydrous solvents were purchased from Sigma Aldrich. Hydrochloric acid and potassium phosphate dibasic were purchased from VWR.

Instrumentation and data processing. ^{1}H, ^{13}C, ^{11}B, and ^{19}F NMRs were acquired on a Varian 400, Varian 500, or Carver B500 spectrometer. Spectra were visualized and analyzed using MestReNova (version 12.0.3). Absorbance spectra were acquired with an Agilent Cary 60 UV-Vis spectrophotometer. Fluorescence traces were acquired with QuantaMaster-400 scanning spectrofluorometer with micro fluorescence quartz cuvettes (Science Outlet). A Nexus 128 Photoacoustic Tomographer (Endra Life Sciences) was used for acquiring photoacoustic data and images. All PA spectra were acquired using continuous rotation mode with a 3 second rotation time. All other images were acquired using continuous rotation mode with a 6 second rotation time. PA data were analyzed using Horos (version 2.2.0) imaging software. No background subtraction was used for any analysis in this work. Statistical analysis was conducted using GraphPad Prism8.

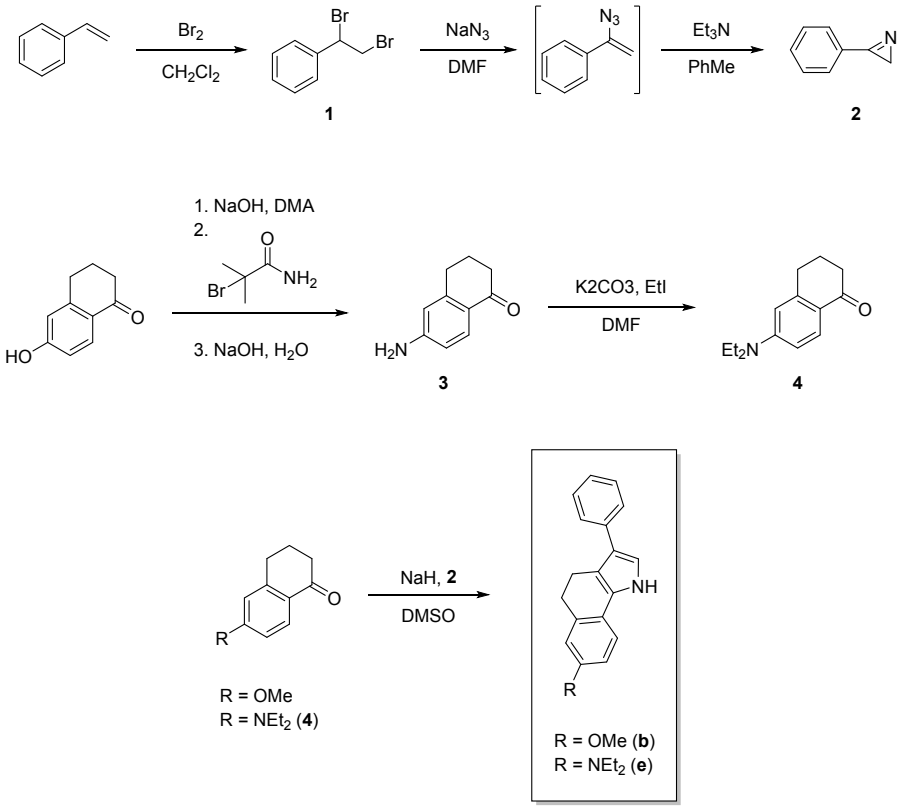
Fluorescence quantum yields. Relative fluorescence quantum yields were measured for all compounds in this study. Main text compounds **1**–**6** (including red-CRaB-HyP), red-HyP-1, rNOD, and CRaB-rNOD were dissolved in $CHCl_3$ and assessed relative to ICG in DMSO (λ_{ex} = 740 nm, λ_{em} = 750 nm – 1000 nm). t-OMe-APC, and CRaB-t-OMe-APC were dissolved in $CHCl_3$ spiked with Et_3N and assessed relative to ICG in DMSO (λ_{ex} = 740 nm, λ_{em} = 750 nm – 1000 nm). rNOD was dissolved in $CHCl_3$ and assessed relative to ICG in DMSO (λ_{ex} = 710 nm, λ_{em} = 720 nm – 1000 nm). HyP-1, CRaB-HyP-1, OMe-APC, CRaB-OMe-APC, photoNOD-1 and CRaB-photoNOD were dissolved in $CHCl_3$ and assessed relative to a symmetrical methoxy-substituted

aza-BODIPY (used by Zhao and Carreira, Chem. Euro. J. 2006. 12:7254-7263) in $CHCl_3$ (λ_{ex} = 650 nm, λ_{em} = 660 nm – 1000 nm).

Region of interest selection and validation. Upon collection of in vivo PA images, regions of interest (ROIs) were selected in order to quantify HyP-1 or CRaB-HyP turn-on under hypoxic conditions. ROIs were characterized by the presence of bright pools of dye, which could be detected as regions that demonstrated substantially higher PA intensity than the surrounding tissue. These regions were identified by visualizing the images with various lower-bound intensity cutoffs (Figure S7, A. 0 – 6000, B. 1000 – 6000, and C. 4000 – 6000 units) to limit the visualization of the blood and surrounding tissue. ROIs were further validated by confirming that under hypoxic conditions, ratiometric turn-on was identified only in the presence of the pooled dye (Figure S8, Table S5). It is worth noting that these visualizations were generated using the Horos software and have no impact upon the measured PA intensities.

Synthetic methods.

Scheme S1. *Preparation of bottom-fused pyrroles* ***b*** *and* ***e***.



(1,2-dibromoethyl)benzene (1). To a 250 mL RBF were added styrene (22.9 mL, 200 mmol, 1 eq.) and dichloromethane (400 mL). The solution was cooled to 0 °C, and bromine (12.3 mL, 240 mmol, 1.2 eq.) was added dropwise via addition funnel. The red reaction mixture was raised to room temperature and stirred for one hour. Upon completion by TLC, the reaction mixture was cooled to 0 °C, diluted with water, and quenched with slow addition of saturated sodium thiosulfate until complete decolorization was noted. The organic layer was dried with sodium sulfate, concentrated to a white powder (51.0 g, 193 mmol, 97% yield) and used without purification.

3-phenyl-2*H*-azirine (2). To a 100 mL RBF were added **1** (2.64 g, 10 mmol, 1.0 eq.), DMF (anhydrous) (40 mL), and sodium azide (1.95 g, 30 mmol, 3.0 eq.). The reaction mixture was stirred vigorously for 5 h. Upon complete consumption of **1** by TLC (hexanes), the reaction mixture was diluted in water and extracted with diethyl ether. The organic layers were washed with brine, concentrated to an oil, and transferred to a preheated RBF containing toluene (15 mL) and triethylamine (2.0 mL). The reaction mixture was heated at 110 °C for 40 min, then cooled rapidly in an ice bath. The crude reaction mixture was loaded directly onto a large pre-packed silica column (5% ethyl acetate/hexanes) and purified with 5% ethyl acetate/hexanes to yield a pale yellow oil (482 mg, 4.1 mmol, 41% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.98 – 7.88 (m, 2H), 7.68 – 7.52 (m, 3H), 1.79 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 165.91, 133.05, 129.71, 129.18, 125.61, 19.82.

6-amino-1-tetralone (3). To a 50 mL RBF were added 6-hydroxy-1-tetralone (811 mg, 5.00 mmol, 1.0 eq.), sodium hydroxide (600 mg, 15.0 mmol, 3.0 eq.) and *N,N*-dimethylacetamide (7.15 mL). The reaction mixture was stirred for 1.5 h at room temperature. Then 2-bromo-2-methylpropanamide (2.49 g, 15.0 mmol, 3.0 eq.) was added and the reaction mixture was stirred at room temperature overnight. Upon consumption of starting material by TLC (50% ethyl acetate/hexanes), indicating complete alkylation, additional NaOH (1.80 g, 45.0 mmol, 9.0 eq.) was added and the reaction mixture was heated to 60 °C for 1 h. Upon completion of the rearrangement reaction by TLC, the reaction mixture was diluted with water (7 mL) and refluxed for 1 h. Upon completion of the hydrolysis by TLC, the reaction mixture was diluted with a further 14 mL water and cooled on an ice bath until product precipitation was noted. The solids were isolated by vacuum filtration, washed with water, and dried. The product was purified by trituration in a 1:1 mixture of hexanes and dichloromethane to yield a beige solid (556 mg, 3.45 mmol, 69%

yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.88 (d, *J* = 8.5 Hz, 1H), 6.53 (dd, *J* = 8.5, 2.3 Hz, 1H), 6.44 – 6.39 (m, 1H), 2.82 (t, *J* = 6.1 Hz, 2H), 2.56 (dd, *J* = 7.1, 5.8 Hz, 2H), 2.06 (p, *J* = 6.4 Hz, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 196.99, 151.36, 147.14, 129.89, 124.17, 113.26, 112.59, 38.98, 30.16, 23.49.

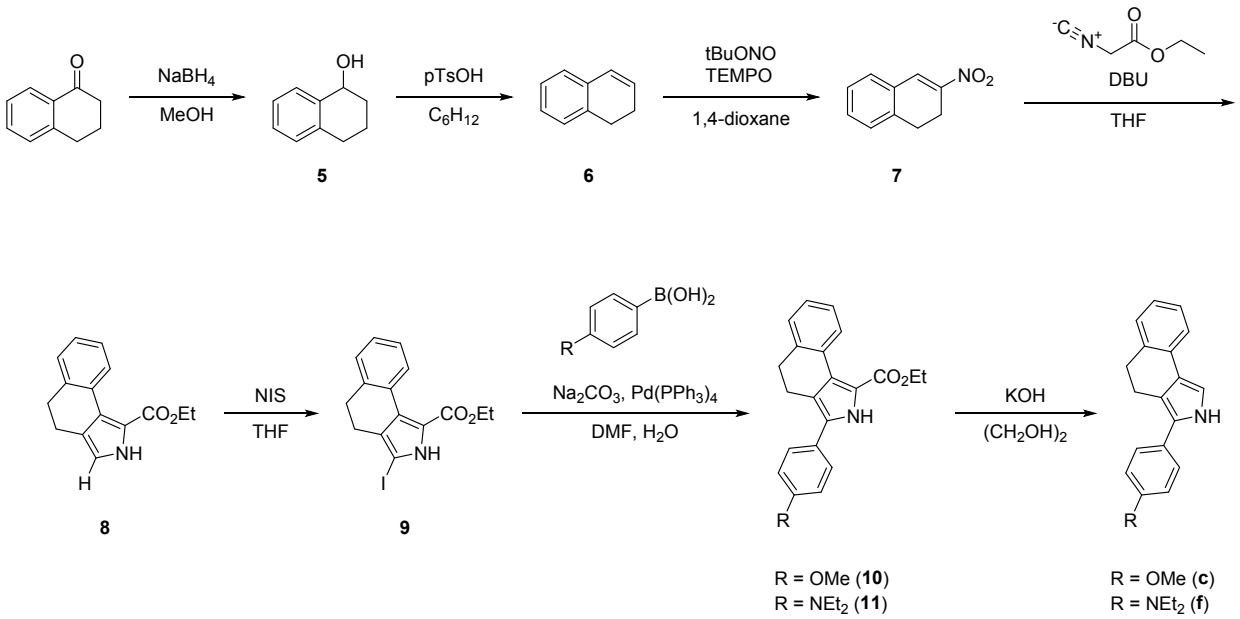
6-diethylamino-1-tetralone (4). To a two-necked RBF equipped with reflux condenser were added **3** (3.00 g, 18.6 mmol, 1.00 eq.), potassium carbonate (5.14 g, 37.2 mmol, 2.0 eq.), and anhydrous DMF (26 mL) under a nitrogen atmosphere. The reaction mixture was heated to 60 °C for 20 min. Ethyl iodide (4.49 mL, 55.8 mmol, 3.0 eq.) was added via syringe, and the reaction was heated overnight (11 h). Additional potassium carbonate (2.57 g, 18.6 mmol, 1.0 eq.) and ethyl iodide (2 mL, 24.8 mmol, 1.3 eq.) was added, and the reaction was heated for an additional 24 h. The reaction mixture was cooled, diluted in water, and extracted with ethyl acetate. The organic layers were washed with brine, dried with sodium sulfate, concentrated, and purified on silica gel by flash chromatography (15% ethyl acetate/hexanes) to yield a yellow-orange oil (2.45 g, 11.3 mmol, 61% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.92 (d, *J* = 8.9 Hz, 1H), 6.55 (dd, *J* = 9.0, 2.6 Hz, 1H), 6.35 (d, *J* = 2.6 Hz, 1H), 3.41 (q, *J* = 7.1 Hz, 4H), 2.85 (t, *J* = 6.1 Hz, 2H), 2.55 (dd, *J* = 7.2, 5.9 Hz, 2H), 2.07 (tt, *J* = 7.1, 5.7 Hz, 2H), 1.20 (td, *J* = 7.1, 1.3 Hz, 7H).^{13}C NMR (126 MHz, $CDCl_3$) δ 196.64, 151.37, 146.91, 129.73, 121.25, 109.86, 108.77, 44.58, 38.94, 30.82, 23.70, 12.71.

7-methoxy-3-phenyl-4,5-dihydro-1*H*-benzo[*g*]indole (pyrrole b). To a 25 mL RBF were added NaH (278 mg, 6.96 mmol, 1.20 eq.) and anhydrous DMSO (5.80 mL) under a nitrogen atmosphere. 6-methoxy-1-tetralone (1.02 g, 5.80 mmol, 1.00 eq.) was added and the reaction was stirred for 30 min at room temperature. The reaction vessel was placed in a cold-water bath and freshly prepared **2** (0.679 g, 5.80 mmol, 1.00 eq.) was added dropwise. The reaction mixture was then stirred for one hour at room temperature. Upon completion by TLC, the reaction mixture was diluted with water and saturated sodium bicarbonate, and the aqueous layer was extracted with diethyl ether. The organic layer was dried over sodium sulfate and purified on silica gel by flash chromatography (10% to 15% ethyl acetate/hexanes). The product was isolated as a beige solid (1.28 g, 4.65 mmol, 80% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.47 – 7.44 (m, 2H), 7.41 – 7.36 (m, 2H), 7.24 (ddt, *J* = 7.7, 6.8, 1.3 Hz, 1H), 7.13 (d, *J* = 8.3 Hz, 1H), 6.92 (dd, *J* = 2.8, 0.8 Hz, 1H), 6.83 (d, *J* = 2.6 Hz, 1H), 6.76 (dd, *J* = 8.3, 2.7 Hz, 1H), 3.82 (d, *J* = 0.8 Hz, 3H), 2.93 (m, 4H). ^{13}C NMR (126

MHz, $CDCl_3$) δ 157.76, 136.98, 136.13, 128.80, 128.64, 127.30, 125.71, 124.05, 122.72, 119.52, 116.31, 115.31, 114.73, 111.52, 55.47, 30.69, 21.33.

***N,N*-diethyl-3-phenyl-4,5-dihydro-1*H*-benzo[g]indol-7-amine (pyrrole d)**. A flame-dried 100 mL RBF was charged with **4** (1.59 g, 7.32 mmol, 1.00 eq.), anhydrous DMSO (45 mL), and sodium hydride (676 mg, 16.9 mmol, 2.31 eq.) and the reaction mixture was stirred for 20 min. **2** (1.19 g, 10.2 mmol, 1.39 eq.) was added dropwise and the reaction was stirred at room temperature overnight. Upon completion by TLC, the reaction mixture was diluted with brine and extracted with ethyl acetate. The organic layers were dried and concentrated to a solid residue, which was recrystallized in ethyl acetate/hexanes. The solids were isolated and the mother liquor was concentrated for further recrystallization, yielding off-white needles (1.54 g from three batches, 4.85 mmol, 66% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.40 (d, J = 7.7 Hz, 2H), 7.31 (t, J = 7.2 Hz, 2H), 7.27 (d, J = 8.3 Hz, 1H), 7.16 – 7.09 (m, 1H), 6.95 (dd, J = 2.8, 1.2 Hz, 1H), 6.55 (s, 1H), 6.50 (d, J = 8.8 Hz, 1H), 3.29 (q, J = 6.8 Hz, 5H), 2.78 (d, J = 3.8 Hz, 2H), 2.48 (dd, J = 3.5, 1.9 Hz, 2H), 1.07 (t, J = 6.6 Hz, 7H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 145.35, 136.62, 135.36, 129.05, 128.40, 126.24, 124.69, 121.78, 120.50, 118.12, 114.81, 113.23, 111.91, 109.68, 43.66, 30.49, 21.40, 12.59.

Scheme S2. *Preparation of top-fused pyrroles* ***c*** *and* ***f****.*



1,2,3,4-tetrahydro-1-naphthol (5). A 1 L RBF was charged with alpha-tetralone (20.0 g, 137 mmol, 1.0 eq.) and anhydrous methanol (274 mL). The solution was cooled to 0 °C and sodium borohydride (6.24 g, 164 mmol, 1.2 eq.) was added portionwise over 15 min. The reaction vessel was brought to room temperature and stirred overnight (12 h) and concentrated. The crude residue was taken up in ethyl acetate and saturated ammonium chloride. The organic layer was dried over sodium sulfate, concentrated, and purified on silica gel by flash column chromatography (gradient, 10% to 50% ethyl acetate/hexanes) to yield a pale amber oil (20.1 g, 136 mmol, 99% yield) which was used without further purification.

1,2-dihydronaphthalene (6). A 250 mL RBF was charged with 1,2,3,4-tetrahydro-1-naphthol (20.1 g, 136 mmol, 1.0 eq.), cyclohexane (108 mL), and pTsOH·H_2O (77 mg, 0.41 mmol, 0.003 eq.). The reaction mixture was stirred at 80 °C for 18 h, cooled, and washed with an aqueous solution of sodium bicarbonate. The aqueous layer was extracted with ethyl acetate, and the combined organic layers were dried over sodium sulfate and concentrated to a crude residue. The crude product was purified by short hexane column to yield a clear oil (9.70 g, 74.5 mmol, 55% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.21 – 7.09 (m, 3H), 7.07 – 7.02 (m, 1H), 6.49 (dt, J = 9.6, 1.9 Hz, 1H), 6.05 (dt, J = 9.1, 4.3 Hz, 1H), 2.83 (t, J = 8.2 Hz, 2H), 2.34 (tdd, J = 8.1, 4.4, 1.8 Hz, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 135.57, 134.24, 128.78, 127.89, 127.64, 126.96, 126.55, 125.99, 27.61, 23.30.

3-nitro-1,2-dihydronaphthalene (7). A large pressure flask was charged with 1,2-dihydronaphthalene (1.74 g, 13.3 mmol, 1.0 eq.), 1,4-dioxane (56 mL), TEMPO (0.857 g, 5.48 mmol, 0.4 eq.), and *tert*-butyl nitrite (3.2 mL, 26.9 mmol, 2.0 eq.). The reaction mixture was stirred at 90 °C for 12 h, cooled, diluted with brine, and extracted with ethyl acetate. The organic layer was dried over sodium sulfate and concentrated. The crude oil was purified on silica gel by flash column chromatography (3% ethyl acetate/hexanes) to yield a viscous oil that slowly crystallized to a pale yellow solid (1.69 g, 9.62 mmol, 72% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.84 (q, J = 1.1 Hz, 1H), 7.40 – 7.20 (m, 4H), 3.06 (dd, J = 9.1, 5.9 Hz, 2H), 3.02 – 2.94 (m, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 147.94, 136.45, 131.61, 131.31, 130.21, 130.14, 127.96, 127.36, 27.95, 22.42.

Ethyl 4,5-dihydro-2*H*-benzo[e]isoindole-1-carboxylate (8). A 100 mL RBF was charged with 3-nitro-1,2-dihydronaphthalene (2.92 g, 16.6 mmol, 1.0 eq.), THF (20 mL), ethyl isocyanoacetate

(1.81 mL, 16.6 mmol, 1.0 eq.), and DBU (2.51 mL, 16.6 mmol, 1.0 eq.) under a nitrogen atmosphere. The reaction mixture was stirred at room temperature for 18 h and formed a thick slurry. The reaction mixture was concentrated and purified on silica gel by flash column chromatography (10% ethyl acetate/hexanes) to yield a clear oil (3.91 g, 16.2 mmol, 98% yield). ^{1}H NMR (500 MHz, CDCl3) δ 9.06 (s, 1H), 8.48 (d, *J* = 7.9 Hz, 1H), 7.32 – 7.28 (m, 1H), 7.25 (d, *J* = 7.3 Hz, 1H), 7.19 (td, *J* = 7.4, 1.4 Hz, 1H), 6.77 (d, *J* = 2.9 Hz, 1H), 4.40 (q, *J* = 7.1 Hz, 2H), 2.87 (dd, *J* = 8.4, 5.7 Hz, 2H), 2.68 (dd, *J* = 8.3, 5.7 Hz, 2H), 1.42 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 161.16, 137.82, 130.97, 128.20, 127.74, 127.02, 126.68, 126.62, 124.38, 117.69, 117.40, 60.64, 31.85, 22.91, 14.36.

Ethyl 3-iodo-4,5-dihydro-2H-benzo[e]isoindole-1-carboxylate (9). A 250 mL RBF was charged with **8** (3.91 g, 16.2 mmol, 1.0 eq.), THF (32 mL), and *N*-iodosuccinimide (4.00 g, 17.8 mmol, 1.1 eq.) under nitrogen atmosphere. The reaction was stirred overnight (12 h) at room temperature, concentrated to a solid, and taken up into ethyl acetate. The organic layer was washed with brine and a 10% solution of sodium sulfite, dried over sodium sulfate, concentrated in ethyl acetate, and triturated in 25% ethyl acetate/hexanes to yield a solid (3.61 g, 9.80 mmol, 61% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.09 (s, 1H), 8.45 (dd, *J* = 7.9, 1.3 Hz, 1H), 7.34 – 7.18 (m,3H), 4.43 (q, *J* = 7.1 Hz, 2H), 2.89 (dd, *J* = 8.2, 6.1 Hz, 2H), 2.62 – 2.53 (m, 2H), 1.44 (t, *J* = 7.2 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 160.06, 137.51, 130.22, 130.08, 128.23, 127.62, 127.48, 126.60, 121.91, 69.89, 60.91, 30.76, 22.41, 14.60.

ethyl 3-(4-methoxyphenyl)-4,5-dihydro-2H-benzo[e]isoindole-1-carboxylate (10). A 25 mL RBF was charged with **9** (109 mg, 0.298 mmol, 1.0 eq.), 4-methoxyphenylboronic acid (73.5 mg, 0.484 mmol, 1.6 eq.), $Pd(PPh_3)_4$ (17.4 mg, 0.0151 mmol, 0.051 eq.), and sodium carbonate (99.9 mg, 0.943 mmol, 3.2 eq.). The flask was evacuated and back-filled with nitrogen three times. Nitrogen-purged DMF (2.25 mL) and deionized water (0.75 mL) were added via syringe, and the reaction mixture was stirred at 150 °C for 6 h, forming a black solution. The reaction mixture was cooled, diluted with brine, and extracted with ethyl acetate. The organic layers were combined, dried, concentrated, and purified on silica gel by flash column chromatography (gradient, 15% ethyl acetate/hexanes to 25% ethyl acetate/hexanes) to yield an off-white solid (84.3 mg, 0.243 mmol, 81% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.99 (s, 1H), 8.47 (dd, *J* = 7.9, 1.3 Hz, 1H), 7.46 – 7.40 (m, 2H), 7.29 (td, *J* = 7.5, 1.6 Hz, 1H), 7.26 – 7.23 (m, 1H), 7.20 (td, *J* = 7.3, 1.4 Hz, 1H),

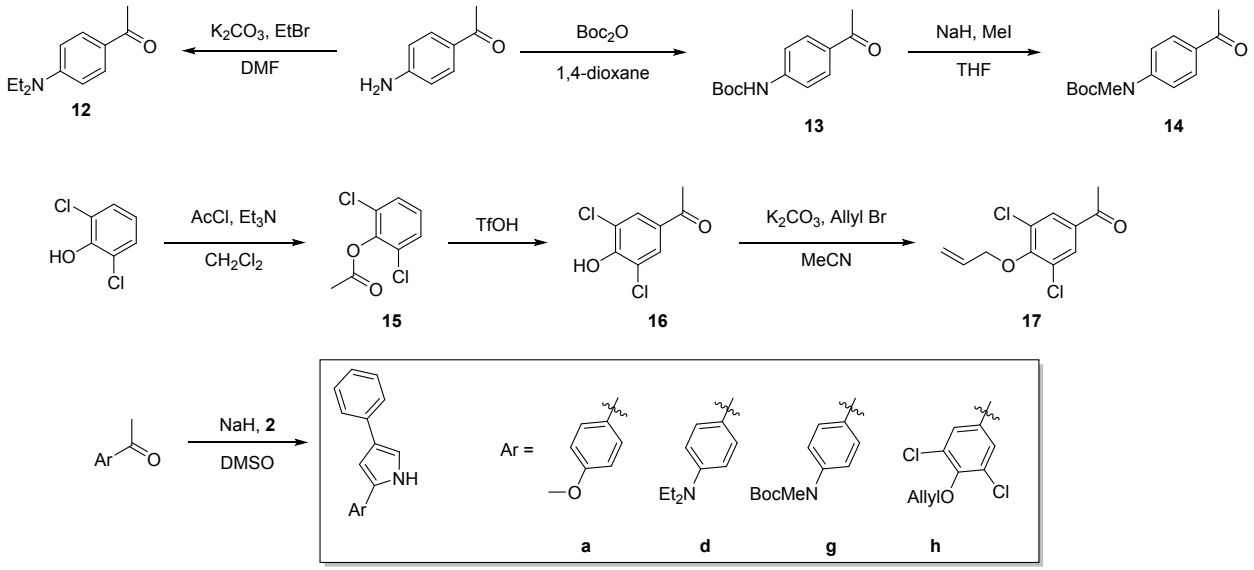
7.03 – 6.98 (m, 2H), 4.41 (q, *J* = 7.1 Hz, 2H), 3.86 (s, 3H), 2.91 – 2.83 (m, 2H), 2.83 – 2.75 (m, 2H), 1.43 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 161.18, 159.42, 137.70, 131.02, 130.69, 128.36, 127.98, 127.92, 127.78, 127.04, 126.48, 124.37, 120.72, 116.52, 114.56, 60.61, 55.55, 31.23, 21.12, 14.69.

3-(4-methoxyphenyl)-4,5-dihydro-2H-benzo[e]isoindole (pyrrole c). A 4 mL vial was charged with **10** (86.9 mg, 0.250 mmol, 1.0 eq.), finely ground potassium hydroxide (70.8 mg, 1.26 mmol, 5.0 eq.), and ethylene glycol (2.5 mL). The vial was sealed and heated to 185 °C for 1 h. The reaction mixture was poured directly into cold water to precipitate the product, which was isolated by vacuum filtration under nitrogen to yield off-white crystals (32.2 mg, 0.117 mmol, 47% yield) which were used without purification.

ethyl 3-(4-(diethylamino)phenyl)-4,5-dihydro-2H-benzo[e]isoindole-1-carboxylate (11). A 25 mL RBF was charged with **9** (95.5 mg, 0.260 mmol, 1.0 eq.), 4-methoxyphenylboronic acid (80.0 mg, 0.414mmol, 1.6 eq.), palladium tetrakis (15.0 mg, 0.0130 mmol, 0.050 eq.), and sodium carbonate (82.0 mg, 0.774 mmol, 3.0 eq.). The flask was evacuated and back-filled with nitrogen three times. Nitrogen-purged DMF (2 mL) and deionized water (0.65 mL) were added via syringe, and the reaction mixture was stirred at 150 °C for 3 h. The reaction mixture was cooled, diluted with brine, and extracted with ethyl acetate. The organic layers were combined, dried, concentrated, and purified on silica gel by flash column chromatography (15% ethyl acetate/hexanes) to yield an off-white powder (74.6 mg, 0.192 mmol, 74% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.06 (s, 1H), 8.46 (d, *J* = 7.7 Hz, 1H), 7.37 (d, *J* = 8.4 Hz, 2H), 7.29 – 7.22 (m, 2H), 7.18 (t, *J* = 7.2 Hz, 1H), 6.76 (d, *J* = 8.4 Hz, 2H), 4.38 (q, *J* = 7.1 Hz, 2H), 3.41 (q, *J* = 7.1 Hz, 4H), 2.89 – 2.74 (m, 4H), 1.41 (t, *J* = 7.2 Hz, 3H), 1.20 (t, *J* = 7.1 Hz, 6H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 161.39, 147.95, 138.29, 132.11, 131.72, 128.53, 128.34, 128.15, 127.24, 126.66, 120.16, 118.75, 116.25, 112.22, 60.82, 44.93, 31.71, 21.68, 14.91, 12.93.

4-(4,5-dihydro-2H-benzo[e]isoindol-3-yl)-*N,N*-dimethylaniline (pyrrole f). A 4 mL vial was charged with **11** (86.9 mg, 0.250 mmol, 1.0 eq.), finely ground potassium hydroxide (70.8 mg, 1.26 mmol, 5.0 eq.), and ethylene glycol (2.5 mL). The vial was sealed and heated to 185 °C for 1 h. The reaction mixture was poured directly into cold water to precipitate the product. The solid was isolated by vacuum filtration under nitrogen to yield off-white crystals (32.2 mg, 0.117 mmol, 47% yield) which were used without purification.

Scheme S3. *Preparation of unfused pyrroles.*



4-diethylaminoacetophenone (12). A 50 mL RBF was charged with 4-aminoacetophenone (1.00 g, 7.50 mmol, 1.0 eq.), potassium carbonate (2.28 g, 16.5 mmol, 2.2 eq.), DMF (7.5 mL), and bromoethane (1.2 mL, 16.5 mmol, 2.2 eq.). The reaction mixture was heated to 60 °C for 24 h. The reaction mixture was cooled, diluted with water, and extracted with diethyl ether. The organic layer was washed with brine, dried over sodium sulfate, and purified on silica gel by flash column chromatography (15% to 30% ethyl acetate/hexanes with 0.1% triethylamine) to obtain a pale oil that slowly solidified (350 mg, 1.83 mmol, 24% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.84 (d, *J* = 9.1 Hz, 2H), 6.61 (d, *J* = 9.1 Hz, 2H), 3.41 (q, *J* = 7.1 Hz, 5H), 2.48 (s, 4H), 1.19 (t, *J* = 7.1 Hz, 8H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 196.18, 151.20, 130.94, 124.70, 110.15, 44.61, 26.00, 12.60.

***tert*-butyl (4-acetylphenyl)carbamate (13).** To a 250 mL round-bottomed flask (RBF) were added 4′-aminoacetophenone (6.7 g, 50 mmol, 1.0 equiv) and di-tert-butyl dicarbonate (13.1 g, 60 mmol, 1.2 equiv). The reaction vessel was capped and flushed with nitrogen. 1,4-Dioxane (60 mL) was added, and the solution was heated to 100 °C for 8.5 h. The reaction was cooled and concentrated to an oil that crystallized to a solid. The solid was washed with 1:3 ethyl acetate/hexanes to give one batch of pure product. The filtrate was then concentrated to a solid and washed with 1:4 ethyl acetate/hexanes to afford a second batch of pure product (total: 10.1 g, 43 mmol, 86% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.89 (d, J = 8.4 Hz, 2H), 7.46 (d, J = 8.3 Hz,

2H), 2.55 (s, 3H), 1.50 (s, 9H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 197.13, 152.35, 143.15, 131.84, 129.94, 117.53, 81.35, 28.37, 26.49

***tert*-butyl (4-acetylphenyl)(methyl)carbamate (14).** To a 250 mL RBF were added **13** (3.0 g, 12.8 mmol, 1.0 equiv) and anhydrous THF (40 mL) under nitrogen. The reaction mixture was cooled to 0 °C, and sodium hydride (60 wt % dispersion in mineral oil) (0.61 g, 15.3 mmol, 1.2 equiv) was added portion-wise over the course of 10 min. After 20 min, methyl iodide (1.57 mL, 31.9 mmol, 2.5 equiv) was added, and the white suspension was warmed to room temperature and stirred until it formed an amber solution. After 40 min at this temperature, completion was noted by TLC (3:17 ethyl acetate/hexanes); the reaction mixture was concentrated, and the crude residue was taken up in ethyl acetate and washed with brine (3×). The organic layer was dried over sodium sulfate and concentrated to a yellow oil (quantitative yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.93 (d, J = 8.8 Hz, 2H), 7.37 (d, J = 8.7 Hz, 2H), 3.32 (s, 3H), 2.59 (s, 3H), 1.49 (s, 9H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 197.10, 154.09, 148.06, 133.42, 128.83, 124.35, 81.14, 36.83, 28.28, 26.52.

2,6-dichlorophenyl acetate (15). A 100 mL RBF was charged with 2,6-dichlorophenol (3.26 g, 20 mmol, 1.0 eq.), dichloromethane (36 mL), and triethylamine (7.0 mL, 50 mmol, 2.5 eq.). The reaction mixture was cooled to 0 °C. Acetyl chloride (1.70 mL, 24 mmol, 1.2 eq.) was added dropwise, and the thick yellow slurry was warmed to room temperature and stirred for 4 h. Upon completion by TLC, the reaction was quenched with aqueous sodium bicarbonate, and the organic layer was dried over sodium sulfate and concentrated to a yellow oil. The product was used without purification.

3,5-dichloro-5-hydroxyacetophenone (16). A 50 mL RBF was charged with **15** (450 mg, 1.55 mmol, 1.0 eq.) and triflic acid (7 mL). The reaction mixture was heated to 40 °C for 18 h, cooled to 0 °C, and basified with saturated sodium carbonate. The solution was washed with ethyl acetate to remove residual starting material. The aqueous layer was then acidified with 1M HCl and extracted with ethyl acetate to isolate product (1.64 g, 8.02 mmol, 40% yield). The product was used without purification.

1-(4-(allyloxy)-3,5-dichlorophenyl)ethan-1-one (17). A 25 mL RBF was charged with **16** (500 mg, 2.44 mmol, 1.0 eq.), potassium carbonate (674 mg, 4.88 mmol, 2.0 eq.), acetonitrile (2.4 mL), and allyl bromide (337 μL, 3.90 mmol, 1.6 eq.) and the reaction mixture was refluxed for 4.5 h. The crude reaction mixture was concentrated and purified on silica gel by flash column

chromatography (10% ethyl acetate/hexanes) to yield a clear oil (397 mg, 1.62 mmol, 66% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.87 (d, *J* = 0.8 Hz, 2H), 6.12 (ddtd, *J* = 17.2, 10.4, 6.0, 0.8 Hz, 1H), 5.47 – 5.35 (m, 1H), 5.29 (dt, *J* = 10.3, 1.2 Hz, 1H), 4.62 (dq, *J* = 6.0, 1.1 Hz, 2H), 2.55 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 194.87, 155.10, 133.98, 132.57, 130.21, 129.13, 119.51, 74.71, 26.54.

2-(4-methoxyphenyl)-4-phenyl-1H-pyrrole (pyrrole a). A 25 mL RBF was charged with 4-methoxyacetophenone (861 mg, 5.73 mmol, 1.0 eq.) and anhydrous DMSO (5.7 mL). Sodium hydride (60% wt in mineral oil) (272 mg, 6.80 mmol, 1.2 eq.) was added and the reaction mixture was stirred at room temperature. After 15 min, **2** (800 mg, 6.83 mmol, 1.2 eq.) was added and the reaction was stirred at room temperature overnight. The reaction mixture was then diluted in brine and extracted with ethyl acetate. The organic layers were combined, dried, and concentrated to a brown solid that was triturated in 3:2 ethyl acetate/hexanes to yield an off-white crystalline solid (421.6 mg, 1.69 mmol, 30% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 11.28 (s, 1H), 7.64 – 7.55 (m, 4H), 7.33 – 7.28 (m, 2H), 7.27 (dd, *J* = 2.7, 1.7 Hz, 1H), 7.11 (ddt, *J* = 8.5, 7.5, 1.2 Hz, 1H), 6.98 – 6.92 (m, 2H), 6.81 (dd, *J* = 2.7, 1.7 Hz, 1H), 3.77 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 158.49, 135.64, 133.13, 128.63, 126.50, 125.66, 125.57, 125.31, 125.17, 114.85, 114.41, 103.00, 55.38.

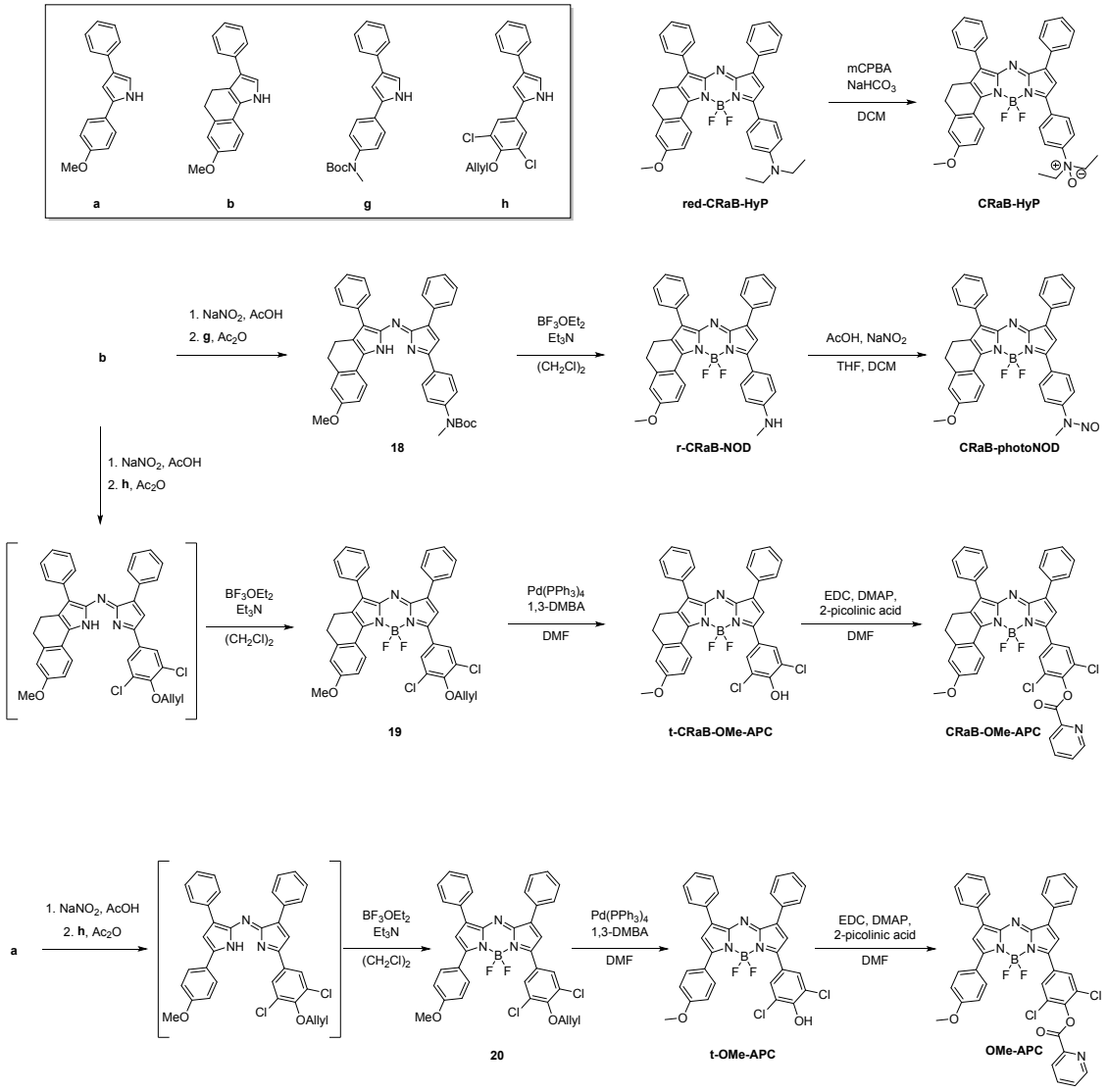
***N,N*-diethyl-4-(4-phenyl-1*H*-pyrrol-2-yl)aniline (pyrrole d)**. A 25 mL RBF was charged with sodium hydride (246 mg, 6.15 mmol, 3.4 eq.) and anhydrous DMSO (1.5 mL). **12** (350 mg, 1.83 mmol, 1.0 eq.) was added at room temperature, and the reaction mixture was stirred for 20 min. **2** (679 mg, 5.8 mmol, 3.4 eq.) was added, and the reaction was stirred at room temperature for 8 h. The reaction mixture was diluted with water and extracted with diethyl ether. The organic layer was washed with brine, dried over sodium sulfate, and concentrated to an orange solid. The solid was purified on neutral alumina by flash column chromatography (15% to 35% ethyl acetate/hexanes) to yield a beige solid (450 mg, 1.55 mmol, 85% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.11 (s, 1H), 7.57 (d, *J* = 7.6 Hz, 2H), 7.47 (d, *J* = 8.3 Hz, 2H), 7.30 (t, *J* = 7.6 Hz, 2H), 7.19 (s, 1H), 7.10 (t, *J* = 7.3 Hz, 1H), 6.67 (m, 3H), 3.42 – 3.27 (m, 4H), 1.09 (t, *J* = 7.0 Hz, 6H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 145.78, 136.09, 133.25, 128.47, 124.80, 124.73, 124.29, 120.21, 114.80, 111.75, 100.43, 43.63, 12.51.

***tert*-butyl methyl(4-(4-phenyl-1H-pyrrol-2-yl)phenyl)carbamate (pyrrole g).** A 25 mL RBF was charged with sodium hydride (48 mg, 1.2 mmol, 1.2 eq.) and anhydrous DMSO (1.0 mL). **14**

(250 mg, 1.0 mmol, 1.0 eq.) was added at room temperature, and the reaction was stirred for 1 h. **2** (140 mg, 1.20 mmol, 1.2 eq.) was added and the reaction was stirred for 1 h. The reaction mixture was diluted with water and extracted with diethyl ether. The organic layer was washed with brine, dried over sodium sulfate, and purified by flash column chromatography (15% to 30% ethyl acetate/hexanes) to yield a pale green oil which was dried under vacuum to yield a yellow foam (132 mg, 0.38 mmol, 38% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.45 (s, 1H), 7.52 – 7.46 (m, 2H), 7.41 – 7.36 (m, 2H), 7.28 (t, *J* = 7.7 Hz, 2H), 7.20 – 7.16 (m, 1H), 7.13 (td, *J* = 7.2, 1.2 Hz, 1H), 7.05 (dq, *J* = 3.8, 2.2 Hz, 1H), 6.71 (dd, *J* = 2.7, 1.6 Hz, 1H), 3.20 (s, 3H), 1.40 (s, 9H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 154.79, 142.11, 135.49, 132.65, 129.68, 128.65, 125.88, 125.74, 125.17, 124.03, 115.54, 103.93, 80.46, 37.28, 28.38, 28.35, 28.30.

2-(4-(allyloxy)-3,5-dichlorophenyl)-4-phenyl-1H-pyrrole (pyrrole h). A 25 mL RBF was charged with sodium hydride (60% wt. in mineral oil) (48 mg, 1.20 mmol, 1.0 eq.) and anhydrous DMSO (1 mL). **17** (245 mg, 1.00 mmol, 1.0 eq.) was added and the reaction was stirred for 40 min. **2** (140 mg, 1.20 mmol, 1.2 eq.) was added and the reaction was stirred for 1 h. The reaction mixture was then diluted in water and extracted with diethyl ether. The organic layer was dried over sodium sulfate, concentrated, and purified on silica gel by flash column chromatography (8% to 15% ethyl acetate/hexanes) to yield a beige solid (230 mg, 0.67 mmol, 67% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.35 (s, 1H), 7.59 – 7.51 (m, 2H), 7.40 (s, 2H), 7.38 (t, *J* = 7.7 Hz, 2H), 7.25 – 7.21 (m, 1H), 7.13 – 7.10 (m, 1H), 6.78 (dd, *J* = 2.8, 1.7 Hz, 1H), 6.18 (ddt, *J* = 16.5, 10.3, 6.0 Hz, 1H), 5.46 (dq, *J* = 17.2, 1.5 Hz, 1H), 5.35 – 5.29 (m, 1H), 4.60 (dt, *J* = 6.0, 1.4 Hz, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 149.43, 135.12, 133.07, 130.32, 130.23, 130.21, 128.87, 127.04, 126.16, 125.29, 124.11, 119.13, 116.62, 105.24, 74.69.

***Scheme S4**. Synthesis of analyte-responsive CRaB probes.*



CRaB-HyP. A 25 mL RBF was charged with red-CRaB-HyP (100 mg, 0.16 mmol, 1.0 eq.), dichloromethane (3.2 mL), and sodium bicarbonate (14.8 mg, 0.176 mmol, 1.1 eq.). The reaction mixture was cooled to 0 °C. mCPBA (77 % wt.) (39.6 mg, 0.176 mmol, 1.1 eq.) was added and the reaction mixture was stirred for 5 min at this temperature before warming to room temperature and stirring for 25 min. The reaction mixture was diluted with a saturated solution of sodium bicarbonate and extracted with ethyl acetate. The organic layer was dried over sodium sulfate and purified by silica column chromatography (10% methanol/dichloromethane) to provide the product (8.0 mg, 0.012 mmol, 8% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.74 (d, J = 9.0 Hz, 1H), 8.16 (d, J = 8.4 Hz, 2H), 8.06 (d, J = 7.6 Hz, 2H), 7.88 (d, J = 8.4 Hz, 2H), 7.74 (d, J = 7.4 Hz,

2H), 7.55 – 7.44 (m, 3H), 7.37 (dt, *J* = 26.4, 7.4 Hz, 3H), 7.03 – 6.98 (m, 1H), 6.96 (s, 1H), 6.85 (d, *J* = 2.6 Hz, 1H), 3.91 (s, 3H), 3.84 (s, 4H), 3.06 – 2.94 (m, 5H), 1.23 (t, *J* = 8.2 Hz, 8H). ^{19}F NMR (471 MHz, CD_2Cl_2) δ -133.85 (dd, *J* = 64.9, 32.2 Hz). ^{11}B NMR (161 MHz, CD_2Cl_2) δ 1.18 (t, *J* = 32.5 Hz).

Compound 18. A RBF was charged with pyrrole **b** (78.1 mg, 0.284 mmol, 1.0 eq.), sodium nitrite (19.8 mg, 0.287 mmol, 1.0 eq.), and acetic acid (2.9 mL). The reaction mixture was stirred at room temperature for 20 min. Pyrrole **g** (99.5 mg, 0.286 mmol, 1.0 eq.) and acetic anhydride (1.2 mL) were added. The reaction mixture was stirred for 20 min at room temperature, then 30 min at 80 °C. The reaction mixture was cooled, diluted with a cold saturated solution of sodium bicarbonate, and filtered to isolate the heterodimer as a dark solid. The resulting solid was purified by alumina column chromatography (3% ethyl acetate/toluene) to yield the product (71.0 mg, 0.112 mmol, 39% yield). ^{1}H NMR (500 MHz, CD_2Cl_2) δ 8.20 (d, *J* = 8.6 Hz, 1H), 8.06 (d, *J* = 7.7 Hz, 2H), 7.76 (d, *J* = 7.5 Hz, 2H), 7.71 (d, *J* = 8.2 Hz, 2H), 7.48 (t, *J* = 7.5 Hz, 2H), 7.41 (t, *J* = 7.6 Hz, 3H), 7.36 (t, *J* = 7.7 Hz, 2H), 7.26 (t, *J* = 7.4 Hz, 1H), 7.05 – 7.01 (m, 1H), 6.97 (s, 1H), 6.89 (d, *J* = 2.5 Hz, 1H), 3.92 (s, 3H), 3.31 (s, 2H), 3.07 (d, *J* = 6.1 Hz, 2H), 3.03 (d, *J* = 6.1 Hz, 2H), 1.49 (s, 9H). ^{13}C NMR (126 MHz, CD_2Cl_2) δ 169.72, 164.90, 163.32, 156.18, 146.51, 145.71, 142.01, 141.01, 139.23, 138.12, 136.36, 135.07, 134.63, 132.05, 130.22, 130.06, 129.81, 129.64, 129.53, 128.81, 127.59, 126.66, 124.39, 115.76, 115.65, 109.22, 82.31, 57.45, 38.85, 31.43, 29.97, 24.61.

r-CRaB-NOD. Compound **18** (31.5 mg, 0.050 mmol, 1.0 eq.) was suspended in dichloroethane (1 mL) and triethylamine (0.12 mL) and BF_3OEt_2 (0.12 mL) were added. The reaction mixture was stirred for 20 min at room temperature and 30 min at 80 °C. The reaction mixture was then cooled, diluted with water, and extracted with dichloromethane. The organic layer was washed with saturated sodium bicarbonate, dried over sodium sulfate, and concentrated. The resulting solid was purified by silica column chromatography (3% ethyl acetate/toluene) to yield the product (18.2 mg, 0.031 mmol, 62% yield). ^{1}H NMR (500 MHz, Acetone-d_6) δ 8.68 (d, *J* = 8.7 Hz, 1H), 8.33 – 8.26 (m, 2H), 8.26 – 8.22 (m, 2H), 7.85 – 7.77 (m, 2H), 7.59 – 7.50 (m, 3H), 7.48 – 7.35 (m, 4H), 7.00 (d, *J* = 8.1 Hz, 2H), 6.82 – 6.76 (m, 2H), 3.93 (s, 3H), 3.02 – 2.92 (m, 7H). ^{13}C NMR (126 MHz, Acetone-d_6) δ 162.70, 158.60, 153.95, 150.74, 146.59, 145.24, 144.94, 142.00, 136.36, 133.48, 133.44, 133.40, 133.36, 131.31, 131.23, 129.85, 129.74, 129.27, 128.98, 128.88, 121.46,

119.60, 119.37, 115.13, 113.60, 112.78, 55.90, 31.12, 30.59, 22.31. ^{19}F NMR (471 MHz, Acetone-d_6) δ -133.83 (dd, J = 66.5, 33.2 Hz). ^{11}B NMR (161 MHz, Acetone-d_6) δ 1.51 (t, J = 33.3 Hz).

CRaB-photoNOD. r-CRaB-NOD (16.4 mg, 0.028 mmol, 1.0 eq.) was dissolved in acetic acid (1 mL), anhydrous THF (2 mL) and dichloromethane (1 mL) and stirred at 0 °C. Sodium nitrite (9.7 mg, 0.14 mmol, 5.0 eq.) was added and the reaction mixture was stirred for 1 h. The reaction mixture was diluted with a saturated solution of sodium bicarbonate and extracted with ethyl acetate. The organic layer was dried over sodium sulfate, filtered through a cotton plug, and concentrated to a green film (8.24 mg, 0.013 mmol, 48% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.75 (d, J = 9.0 Hz, 1H), 8.21 – 8.15 (m, 2H), 8.10 – 8.04 (m, 2H), 7.77 – 7.73 (m, 2H), 7.73 – 7.68 (m, 2H), 7.51 (dd, J = 8.3, 6.6 Hz, 2H), 7.49 – 7.44 (m, 1H), 7.43 – 7.38 (m, 2H), 7.37 – 7.33 (m, 1H), 6.98 (d, J = 8.0 Hz, 2H), 6.85 (d, J = 2.6 Hz, 1H), 3.91 (s, 3H), 3.52 (d, J = 0.7 Hz, 3H), 3.02 – 2.93 (m, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 163.61, 157.59, 151.93, 147.69, 145.91, 144.04, 142.90, 139.64, 139.54, 133.60, 133.13, 132.97, 131.68, 130.66, 130.63, 130.59, 129.00, 128.70, 128.63, 128.59, 128.39, 119.68, 118.57, 116.76, 114.83, 113.66, 55.75, 31.01, 30.59, 22.06. ^{19}F NMR (471 MHz, $CDCl_3$) δ -133.75 (dd, J = 64.9, 32.5 Hz). ^{11}B NMR (161 MHz, $CDCl_3$) δ 1.23 (t, J = 32.6 Hz).

Compound 19. A RBF was charged with pyrrole **b** (156 mg, 0.567 mmol, 1.0 eq.), sodium nitrite (39.2 mg, 0.568 mmol, 1.0 eq.), and acetic acid (6.5 mL). The reaction mixture was stirred at room temperature fore 20 min. Pyrrole **h** (195 mg, 0.568 mmol, 1.0 eq.) and acetic anhydride (2.6 mL) were added. The reaction mixture was stirred for 20 min at room temperature, then 30 min at 80 °C. The reaction mixture was cooled, diluted with a cold saturated solution of sodium bicarbonate, and filtered to isolate the heterodimer as a dark solid. The resulting solid was suspended in dichloroethane (6 mL) and triethylamine (680 μL) and BF_3OEt_2 (680 μL) were added. The reaction mixture was stirred for 20 min at room temperature and 30 min at 80 °C. The reaction mixture was then cooled, diluted with water, and extracted with ethyl acetate. The organic layer was washed with saturated sodium bicarbonate, dried over sodium sulfate, and concentrated. The resulting solid was purified by alumina column chromatography (50% dichloromethane/hexanes and then 75% dichloromethane/hexanes) to yield the product (132.5 mg, 0.195 mmol, 30% yield over 2 steps). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.75 (d, J = 9.0 Hz, 1H), 8.08 – 8.01 (m, 2H), 8.00 – 7.96 (m, 2H), 7.75 – 7.70 (m, 2H), 7.53 – 7.43 (m, 3H), 7.42 – 7.30 (m, 3H), 7.03 – 6.97 (m, 1H), 6.87 – 6.82

(m, 2H), 6.29 – 6.14 (m, 1H), 5.51 (dp, J = 17.3, 1.4 Hz, 1H), 5.34 (dt, J = 10.4, 1.3 Hz, 1H), 4.67 (dq, J = 6.1, 1.3 Hz, 2H), 3.93 – 3.87 (m, 3H), 2.95 (dp, J = 10.8, 6.0, 5.4 Hz, 4H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 163.99, 158.68, 151.96, 148.98, 148.07, 146.23, 143.44, 139.83, 138.83, 134.10, 133.36, 133.17, 133.04, 131.44, 130.55, 130.37, 129.66, 129.63, 129.58, 129.09, 128.90, 128.59, 128.35, 119.35, 118.90, 116.42, 114.97, 113.67, 74.66, 55.77, 30.45, 22.02. ^{19}F NMR (471 MHz, $CDCl_3$) δ -133.66 (dd, J = 64.8, 31.9 Hz). ^{11}B NMR (161 MHz, $CDCl_3$) δ 1.10 (t, J = 32.4 Hz).

t-CRaB-OMe-APC. A two-neck RBF was charged with compound **19** (34 mg, 0.050 mmol, 1.0 eq.), 1,3-dimethylbarbituric acid (10.2 mg, 0.065 mmol, 1.3 eq.) and $Pd(PPh_3)_4$ (11.1 mg, 0.0096 mmol, 0.2 eq.). The solids were dried under vacuum and flushed with nitrogen. Anhydrous DMF (1 mL) was added and the reaction mixture was stirred for 1 h. The reaction mixture was poured into brine and extracted with ethyl acetate. The organic layer was dried, concentrated, and purified by silica column chromatography (0.1% AcOH in dichloromethane) to yield the product (26.1 mg, 0.041 mmol, 82% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.56 (d, J = 8.9 Hz, 1H), 8.13 (d, J = 9.8 Hz, 4H), 7.78 (d, J = 7.6 Hz, 2H), 7.58 (t, J = 7.5 Hz, 2H), 7.51 (t, J = 7.4 Hz, 1H), 7.43 (dd, J = 13.0, 5.5 Hz, 3H), 7.37 (t, J = 7.3 Hz, 1H), 7.13 (d, J = 9.7 Hz, 2H), 3.92 (s, 3H), 2.98 (tt, J = 13.7, 8.3 Hz, 4H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 163.69, 156.78, 150.70, 149.29, 146.90, 146.68, 142.82, 138.45, 138.27, 134.22, 132.32, 131.82, 130.82, 130.22, 129.20, 129.10, 128.78, 128.66, 128.53, 128.35, 124.40, 122.34, 118.48, 117.53, 114.84, 114.01, 55.93, 29.39, 21.15. ^{19}F NMR (471 MHz, DMSO-d_6) δ -132.02 (dd, J = 66.3, 31.4 Hz). ^{11}B NMR (161 MHz, DMSO-d_6) δ 1.10 (t, J = 32.9 Hz).

CRaB-OMe-APC. A 25 mL RBF was charged with t-CRaB-OMe-APC (100 mg, 0.157 mmol, 1.0 eq.), 2-picolinic acid (77.5 mg, 0.630 mmol, 4.0 eq.), 4-(dinmethylamino)pyridine (3.4 mg, 0.0278 mmol, 0.18 eq.), EDC-HCl (46.1 mg, 0.241 mmol, 1.5 eq.) and anhydrous DMF (7.8 mL). The reaction mixture was stirred at room temperature for 1 h. The reaction was diluted with brine and extracted with ethyl acetate. The organic layer was washed with brine and 1% HCl, dried over sodium sulfate, and purified by column chromatography (gradient, 30% to 60% to 100% ethyl acetate/hexanes with 0.1% AcOH) to provide the product (34.0 mg, 0.0457 mmol, 29% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.92 (ddd, J = 4.8, 1.8, 0.9 Hz, 1H), 8.77 (d, J = 9.1 Hz, 1H), 8.40 – 8.35 (m, 1H), 8.09 – 8.02 (m, 4H), 7.97 (td, J = 7.7, 1.8 Hz, 1H), 7.76 – 7.71 (m, 2H), 7.61 (ddd, J = 7.8, 4.7, 1.2 Hz, 1H), 7.54 – 7.49 (m, 2H), 7.49 – 7.43 (m, 1H), 7.39 (dd, J = 8.3, 6.5 Hz, 2H),

7.36 – 7.31 (m, 1H), 7.03 (dd, J = 9.1, 2.7 Hz, 1H), 6.88 (s, 1H), 6.85 (d, J = 2.7 Hz, 1H), 3.92 (s, 3H), 3.04 – 2.90 (m, 4H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 164.25, 161.69, 159.36, 150.62, 148.42, 148.14, 146.50, 146.41, 144.55, 143.37, 140.14, 138.63, 137.43, 134.43, 133.78, 133.12, 132.97, 131.41, 130.60, 129.47, 129.21, 128.94, 128.86, 128.64, 128.62, 128.42, 127.93, 126.47, 119.29, 116.51, 115.15, 113.77, 55.85, 30.50, 22.12. ^{19}F NMR (471 MHz, $CDCl_3$) δ -133.62 (dd, J = 65.0, 32.1 Hz).^{11}B NMR (161 MHz, $CDCl_3$) δ 1.10 (t, J = 32.4 Hz).

Compound 20 A RBF was charged with pyrrole **a** (91.8 mg, 0.368 mmol, 1.0 eq.), sodium nitrite (24.7 mg, 0.358 mmol, 1.0 eq.), and acetic acid (3.6 mL). The reaction mixture was stirred at room temperature fore 20 min. Pyrrole **h** (125 mg, 0.363 mmol, 1.0 eq.) and acetic anhydride (1.4 mL) were added. The reaction mixture was stirred for 20 min at room temperature, then 30 min at 80 °C. The reaction mixture was cooled, diluted with a cold saturated solution of sodium bicarbonate, and filtered to isolate the heterodimer as a dark solid. The solid was suspended in dichloroethane (7 mL) and triethylamine (850 μL) and BF_3OEt_2 (850 μL) were added. The reaction mixture was stirred for 20 min at room temperature and 30 min at 80 °C. The reaction mixture was then cooled, diluted with water, and extracted with dichloromethane. The organic layer was washed with saturated sodium bicarbonate, dried over sodium sulfate, and concentrated. The resulting solid was purified by alumina column chromatography (50% dichloromethane/hexanes) to yield the product (53.5 mg, 0.0789 mmol, 21% yield over 2 steps). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.21 – 8.12 (m, 2H), 8.09 – 8.05 (m, 2H), 8.05 – 8.02 (m, 2H), 7.97 (s, 2H), 7.51 – 7.37 (m, 7H), 7.16 (s, 1H), 7.09 – 7.00 (m, 2H), 6.90 (s, 1H), 6.28 – 6.11 (m, 1H), 5.48 (dt, J = 17.0, 1.5 Hz, 1H), 5.33 (dd, J = 10.4, 1.5 Hz, 1H), 4.64 (dt, J = 6.0, 1.3 Hz, 2H), 3.91 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 163.17, 162.07, 152.57, 152.32, 147.12, 145.74, 144.23, 141.91, 133.07, 132.69, 132.51, 131.94, 130.06, 129.83, 129.79, 129.71, 129.62, 129.33, 129.29, 128.81, 128.75, 123.28, 120.28, 119.03, 117.73, 114.72, 74.72, 55.72. ^{19}F NMR (471 MHz, $CDCl_3$) δ -131.70 (dd, J = 63.5, 31.7 Hz). ^{11}B NMR (161 MHz, $CDCl_3$) δ 0.95 (t, J = 31.7 Hz).

t-OMe-APC. A two-neck RBF was charged with compound **20** (42.8 mg, 0.0656 mmol, 1.0 eq.), 1,3-dimethylbarbituric acid (12.8 mg, 0.082 mmol, 1.3 eq.) and $Pd(PPh_3)_4$ (15.2 mg, 0.0131 mmol, 0.2 eq.). The solids were dried under vacuum and flushed with nitrogen. Anhydrous DMF (1 mL) was added and the reaction mixture was stirred for 1 h. The reaction mixture was poured into brine and extracted with ethyl acetate. The organic layer was dried, concentrated, and purified by silica

column chromatography (1% methanol/dichloromethane) to yield the product (29.8 mg, 0.0467 mmol, 71% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.26 – 8.21 (m, 2H), 8.21 – 8.13 (m, 6H), 7.71 (s, 1H), 7.64 (s, 1H), 7.58 – 7.44 (m, 7H), 7.20 – 7.14 (m, 2H), 3.91 (s, 3H). ^{19}F NMR (471 MHz, DMSO-d_6) δ -130.15 (dd, J = 65.8, 32.4 Hz). ^{11}B NMR (161 MHz, DMSO-d_6) δ 0.97 (t, J = 32.8 Hz).

OMe-APC. 25 mL RBF was charged with t-OMe-APC (22.9 mg, 0.037 mmol, 1.0 eq.), 2-picolinic acid (18.4 mg, 0.150 mmol, 4.0 eq.), 4-(dinmethylamino)pyridine (0.46 mg, 0.0307 mmol, 0.1 eq.), EDC-HCl (10.8 mg, 0.056 mmol, 1.5 eq.) and anhydrous DMF (2 mL). The reaction mixture was stirred at room temperature for 4 h. The reaction was diluted with brine and extracted with ethyl acetate. The organic layer was dried over sodium sulfate, and purified by column chromatography (10% ethyl acetate/toluene) to yield the product (25.7 mg, .0358 mmol, 97% yield). ^{1}H NMR (499 MHz, $CDCl_3$) δ 8.92 (dt, J = 4.4, 1.5 Hz, 1H), 8.36 (dt, J = 7.8, 1.1 Hz, 1H), 8.20 (d, J = 9.0 Hz, 2H), 8.11 – 8.01 (m, 6H), 8.00 – 7.94 (m, 1H), 7.62 (ddd, J = 7.7, 4.7, 1.2 Hz, 1H), 7.51 – 7.38 (m, 7H), 7.20 (s, 1H), 7.06 (dd, J = 9.7, 2.7 Hz, 2H), 6.94 (s, 1H), 3.91 (d, J = 6.1 Hz, 4H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 163.41, 162.85, 161.61, 151.22, 150.62, 147.50, 146.38, 146.14, 145.07, 144.04, 141.55, 137.45, 132.80, 132.74, 132.33, 131.82, 130.19, 129.67, 129.57, 129.33, 129.25, 129.06, 128.83, 128.77, 127.98, 126.49, 123.06, 117.69, 117.68, 114.83, 55.75. ^{19}F NMR (471 MHz, $CDCl_3$) δ -131.50 (dd, J = 63.6, 31.7 Hz). ^{11}B NMR (161 MHz, $CDCl_3$) δ 0.96 (t, J = 31.7 Hz).

Supplementary Figures and Tables

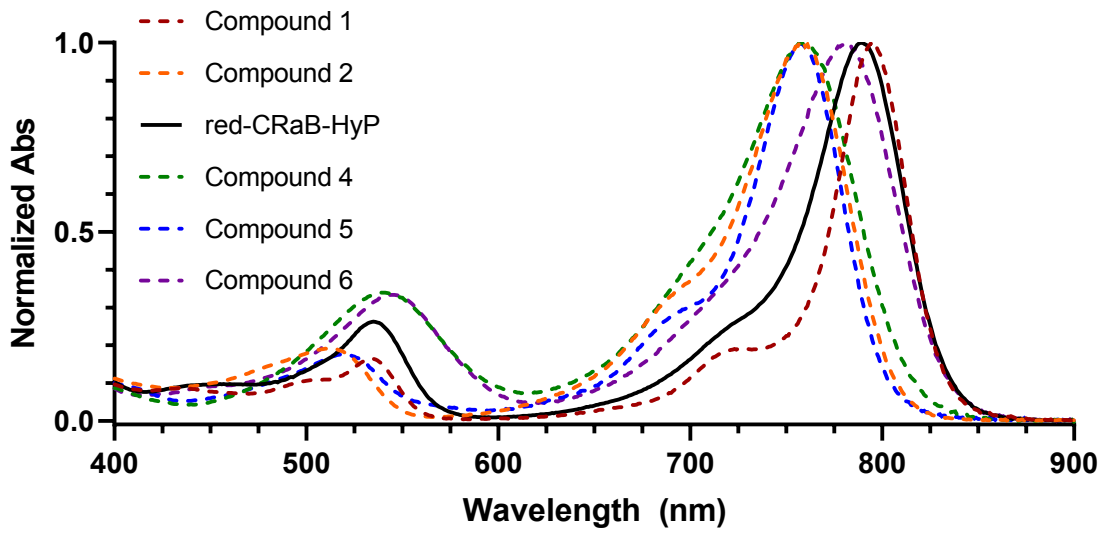


Figure S1. Normalized absorbance spectra of red-HyP-1 analogues in $CHCl_3$.

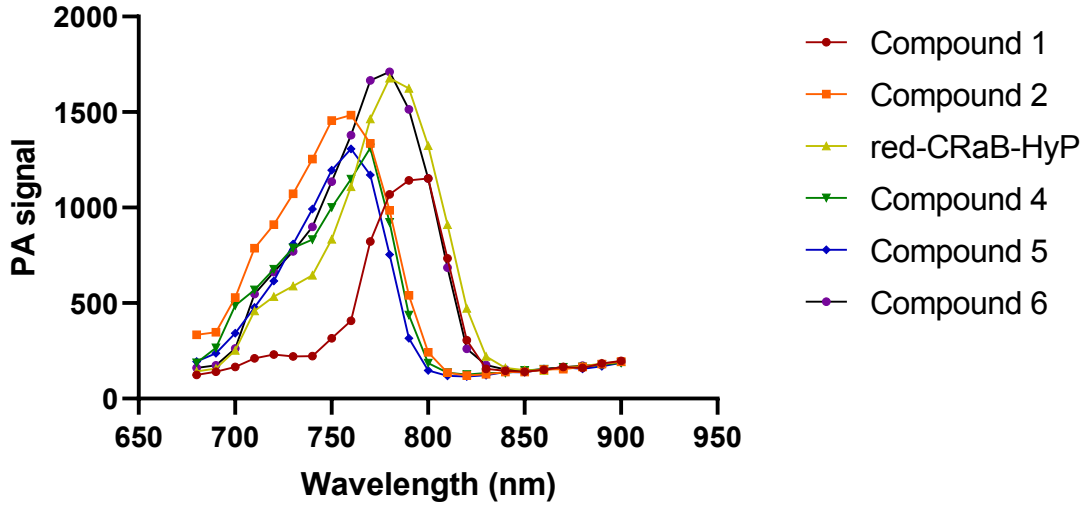


Figure S2. PA spectra for red-HyP-1 analogues with various conformational restrictions. Spectra were acquired for 10 μM solutions in $CHCl_3$, in tissue-mimicking phantoms.

Table S1. PA wavelengths for the red-HyP analogues (main text compounds **1**–**6**) in chloroform, as shown above in Figure S2.

Compound	λabs (nm)	λPA (nm)
red-HyP-1	760	770
1	796	790
2	759	760
3	789	780
4	757	770
5	759	760
6	782	780

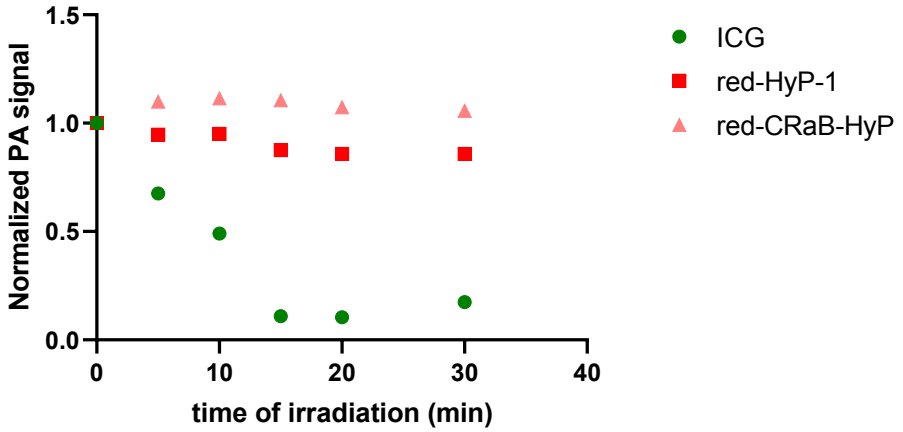


Figure S3. Photostability of red-CRaB-HyP relative to ICG and red-HyP-1. Photostability was assessed in $CHCl_3$ in tissue-mimicking phantoms.

Table S2. Absorbance wavelengths for the red-HyP analogues (main text compounds **1**–**6**) in 1 mL of 1:1 acetonitrile/PBS, and shift in wavelengths upon protonation with 20 µL HCl.

Compound	λ (nm) at pH 7.4	λ (nm) upon protonation	Δλ
red-HyP-1	760	665	95
1	792	713	79
2	751	695	56
3 (red-CRaB-HyP)	794	679	115
4	761	667	94
5	751	693	58
6	780	683	97

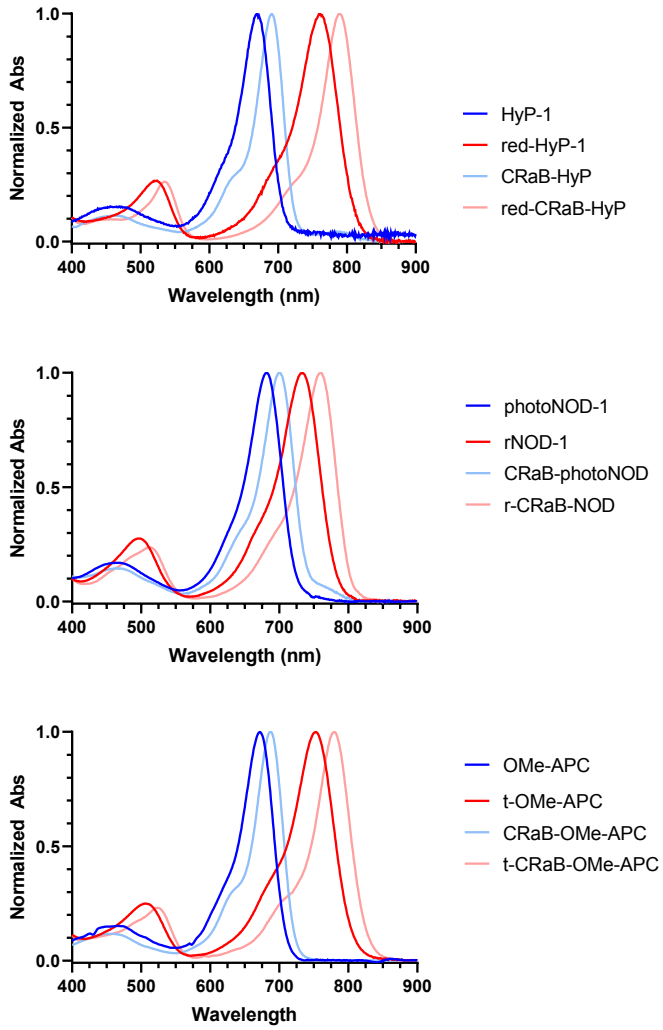


Figure S4. Normalized absorbance spectra of probe/product pairs in $CHCl_3$. Note t-OMe-APC and t-CRaB-OMe-APC spectra were obtained in the presence of Et_3N.

Table S3. PA wavelengths λ_{red} *and* λ_{blue} for each probe-product pair in chloroform and in 1:1 acetonitrile/PBS mixtures, as shown in Figure S5.

Probe	Product	$CHCl_3$		1:1 MeCN/PBS	
		λ_{blue}	λ_{red}	λ_{blue}	λ_{red}
HyP-1	red-HyP-1	680	770	680	770
photoNOD-1	rNOD-1	680	730	680	740
OMe-APC	t-OMe-APC	680	750	680	750
CRaB-HyP	red-CRaB-HyP	690	780	680	790
CRaB-photoNOD	r-CRaB-NOD	700	760	680	770
CRaB-OMe-APC /	t-CRaB-OMe-APC	680	780	680	780

Table S4. $\Delta\lambda_{abs}$ for each probe-product pair in chloroform.

	Unrestricted λ_{abs} (nm)	CRaB λ_{abs} (nm)
redHyP / HyP	90	98
rNOD / photoNOD	52	61
tOMe-APC / OMe-APC	81	93

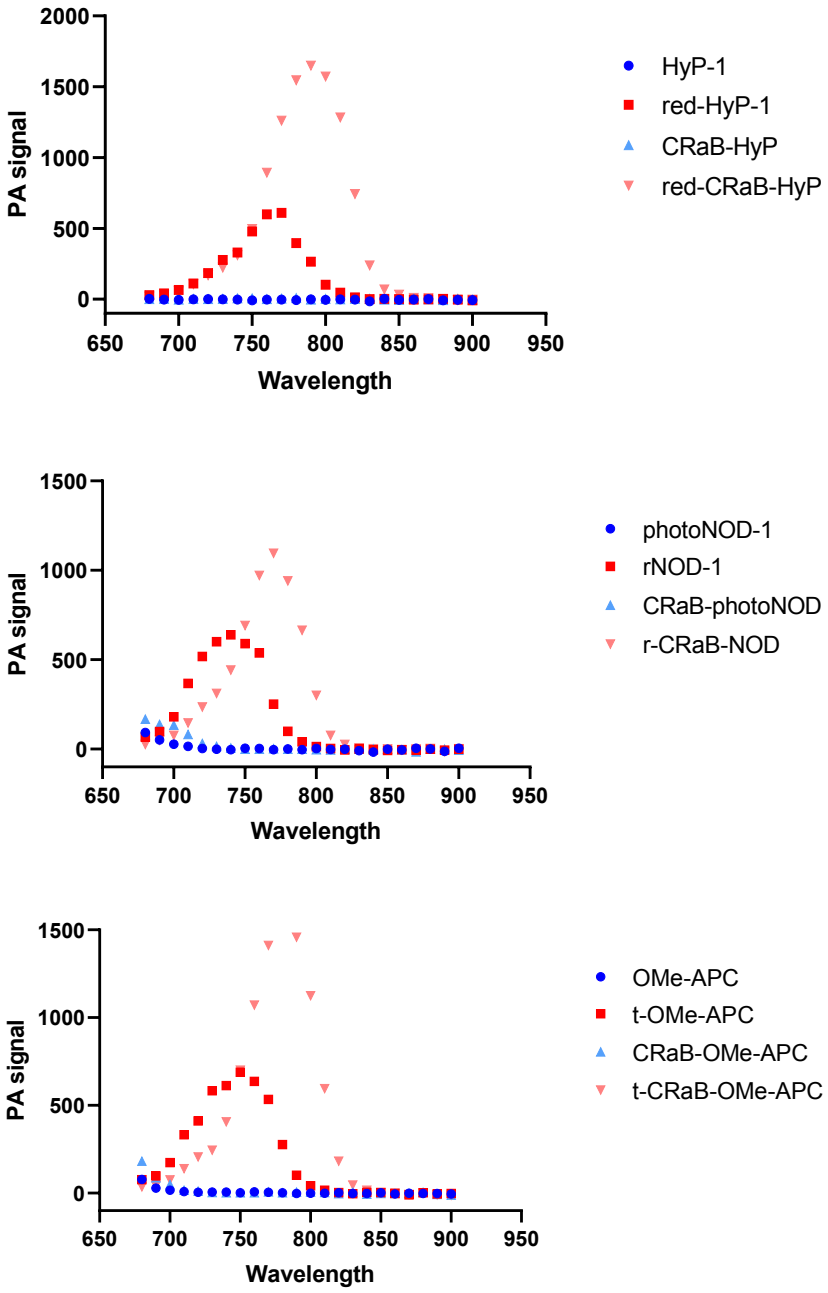


Figure S5. PA spectra of HyP, photoNOD, and APC compounds in 1:1 acetonitrile/PBS mixtures.

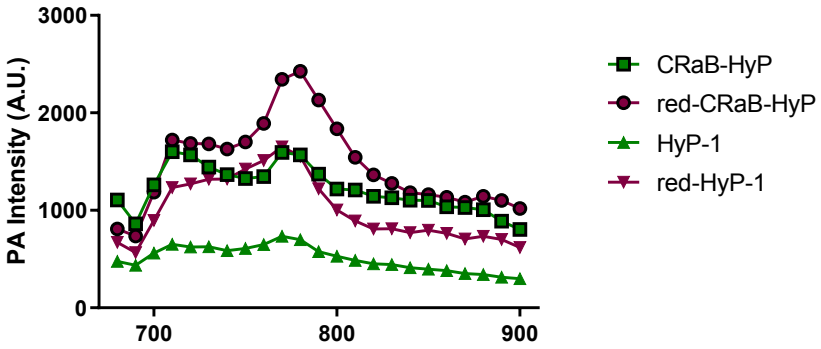


Figure S6. *In vivo* PA spectra of HyP-1, red-HyP-1, CRaB-HyP, and red-CRaB-HyP in subcutaneous space, 1 h post-injection. 710 nm and 770 nm were selected as λ_{blue} and λ_{red}, respectively.

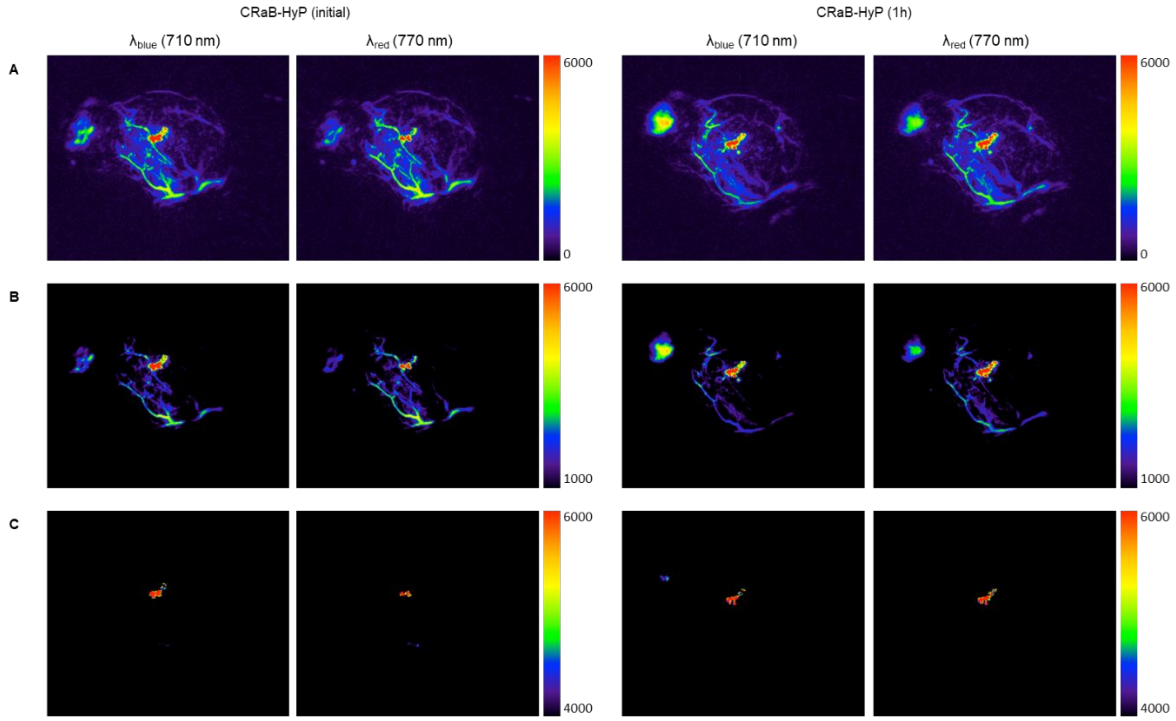


Figure S7. Representative PA images of tumors after injection of CRaB-HyP. Regions of interest containing pooled dye can be identified by visualization of only high intensity PA signals.

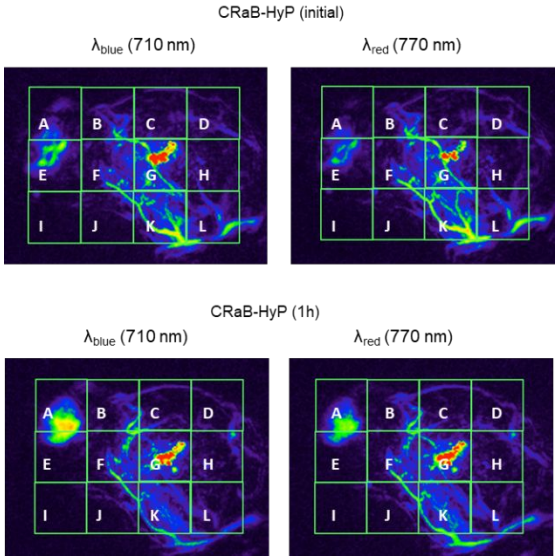


Figure S8. Sample grid analysis of PA images shown in Figure S7 validates that ratiometric fold turn-on is probe dependent. PA intensities are quantified below in Table S5.

Table S5. Sample quantitative analysis of PA intensities (as shown in Figure S2) showing selective ratiometric turn-on in the region of interest.

	Initial			1h			Ratiometric turn-on
	710 nm	770 nm	Ratio	710 nm	770nm	Ratio	
A	456.96	352.51	0.77	1083.71	773.99	0.71	0.93
B	496.09	526.28	1.06	497.74	502.88	1.01	0.95
C	409.49	388.84	0.95	423.21	453.31	1.07	1.13
D	375.84	378.02	1.01	340.14	370.99	1.09	1.08
E	777.74	624.13	0.80	477.45	405.11	0.85	1.06
F	719.88	782.76	1.09	773.71	754.84	0.98	0.90
G	1756.87	1442.86	0.82	1470.17	1662.40	1.13	1.38
H	393.23	399.63	1.02	418.50	439.94	1.05	1.03
I	217.58	201.45	0.93	200.07	207.67	1.04	1.12
J	305.12	309.81	1.02	347.60	358.53	1.03	1.02
K	1343.21	1466.64	1.09	1001.67	1210.96	1.21	1.11
L	738.61	800.74	1.08	495.74	621.75	1.25	1.16

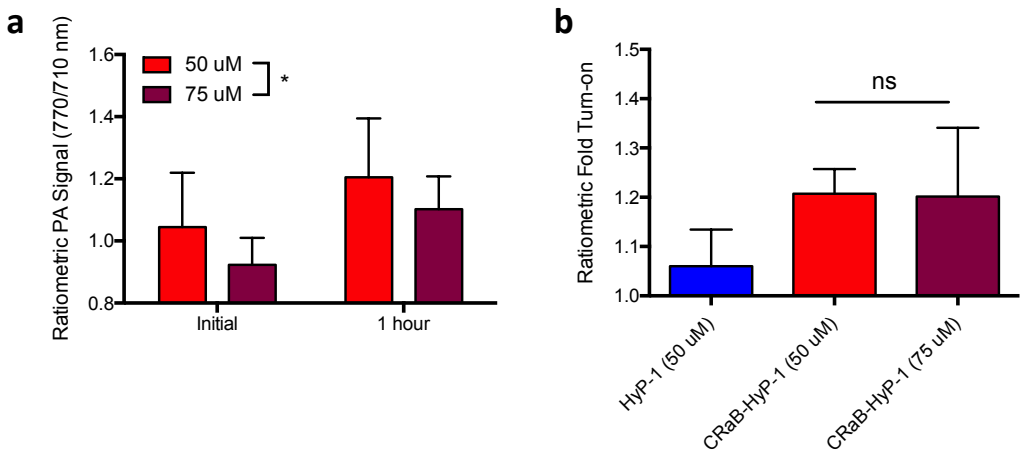


Figure S9. Dose dependence of ratiometric PA imaging with CRaB-HyP. a) Time and concentration dependence of ratiometric PA signal in hypoxic tumors. Signal ratio is consistently lower when higher dose is used, but still increases over time as expected. Data presented as mean ± S.D. (n ≥ 6). Statistical analysis performed using 2-way ANOVA. b) Ratiometric fold turn-on of HyP-1 and multiple concentrations of CRaB-HyP. Ratiometric fold turn-on of CRaB-HyP is consistent across multiple doses. Data presented as mean ± S.D. (n ≥ 6). Statistical analysis performed using student's t-test. *$p < 0.05$; ns = not significant.

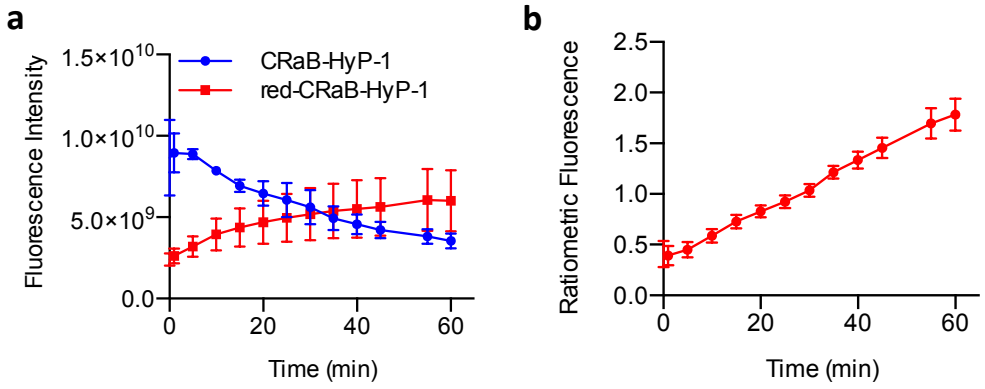


Figure S10. Fluorescence imaging of CRaB-HyP turnover *in vivo* following intratumoral injection (50 µL, 50 µM) of CRaB-HyP. Images were acquired at the indicated time points using excitation/emission filter sets of 675/720 nm (detection of CRaB-HyP) and 745/820 nm (detection of red-CRaB-HyP). a) Time-dependent change in tumor fluorescence intensity for individual filter sets. b) Time-dependent increase in fluorescence ratio (red-CRaB-HyP/CRaB-HyP). Data presented as mean ± S.D. (n=3).

NMR Spectra of red-HyP analogues (Main Text, Compounds 1-6):

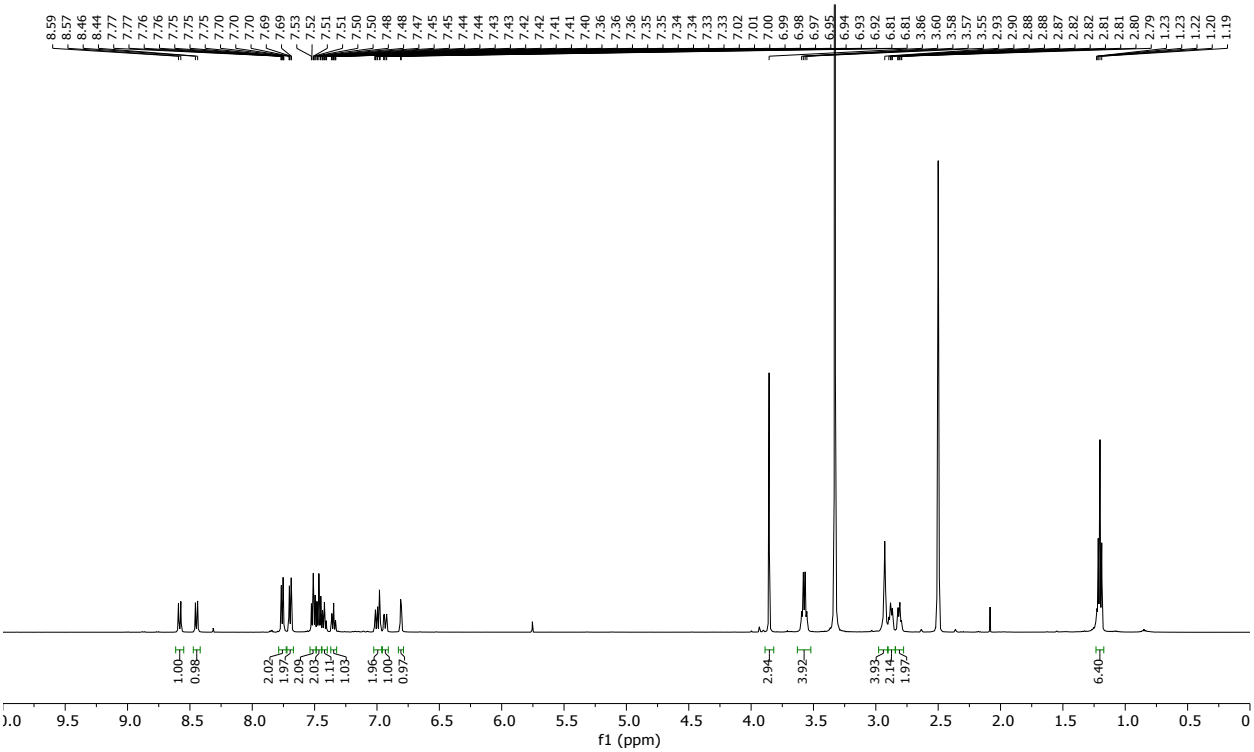


Figure S11. ^{1}H NMR of red-HyP analogue **1** (DMSO-d_6).

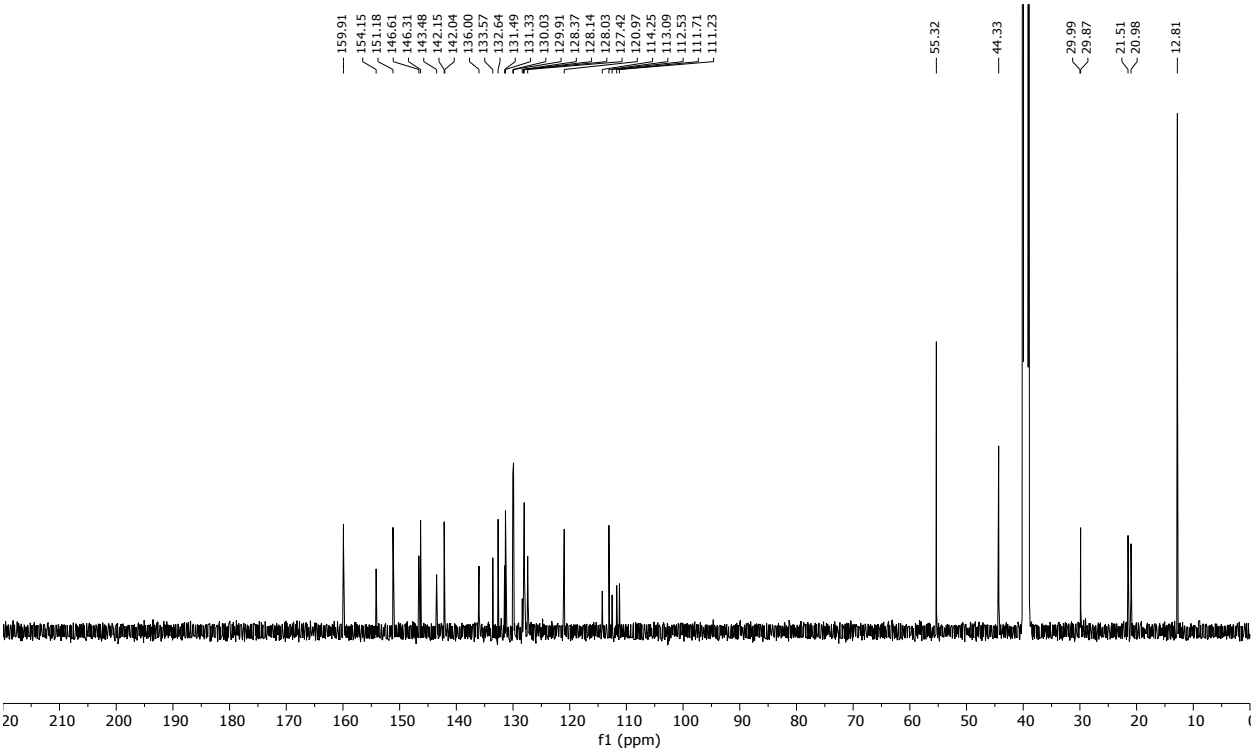


Figure S12. ^{13}C NMR of red-HyP analogue **1** (DMSO-d_6).

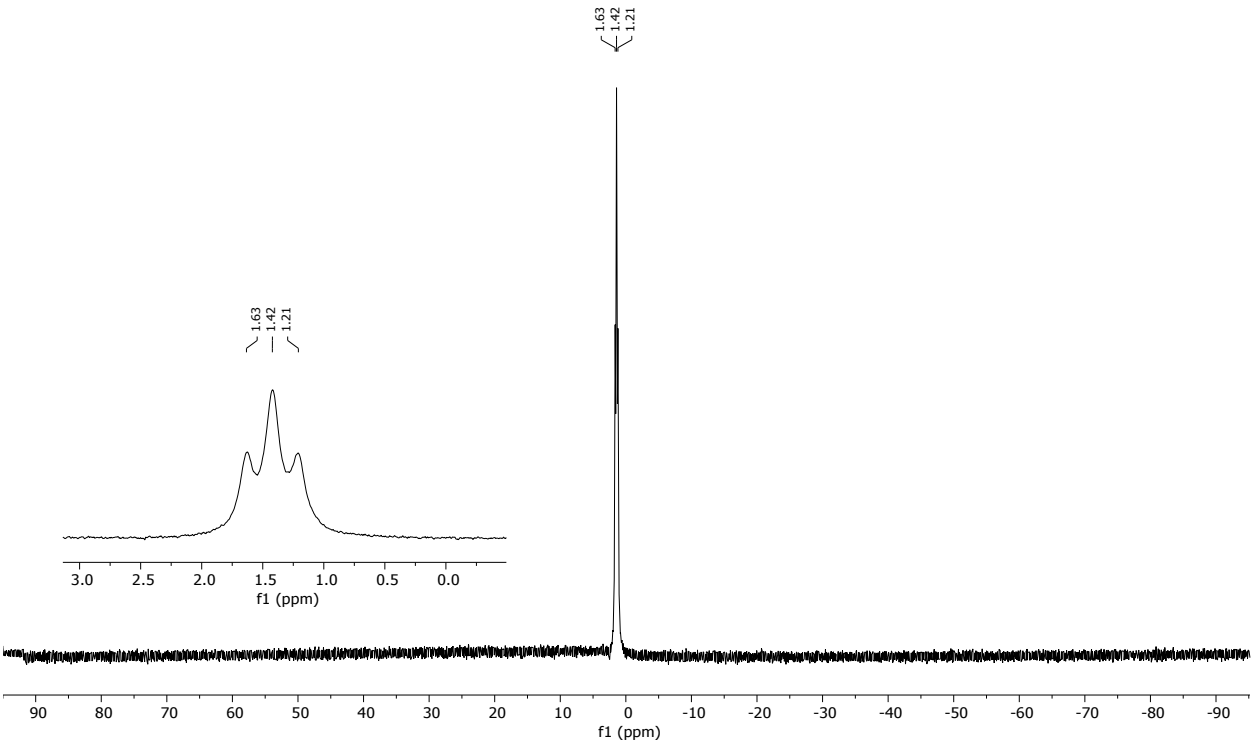


Figure S13. ^{11}B NMR of red-HyP analogue **1** (DMSO-d_6).

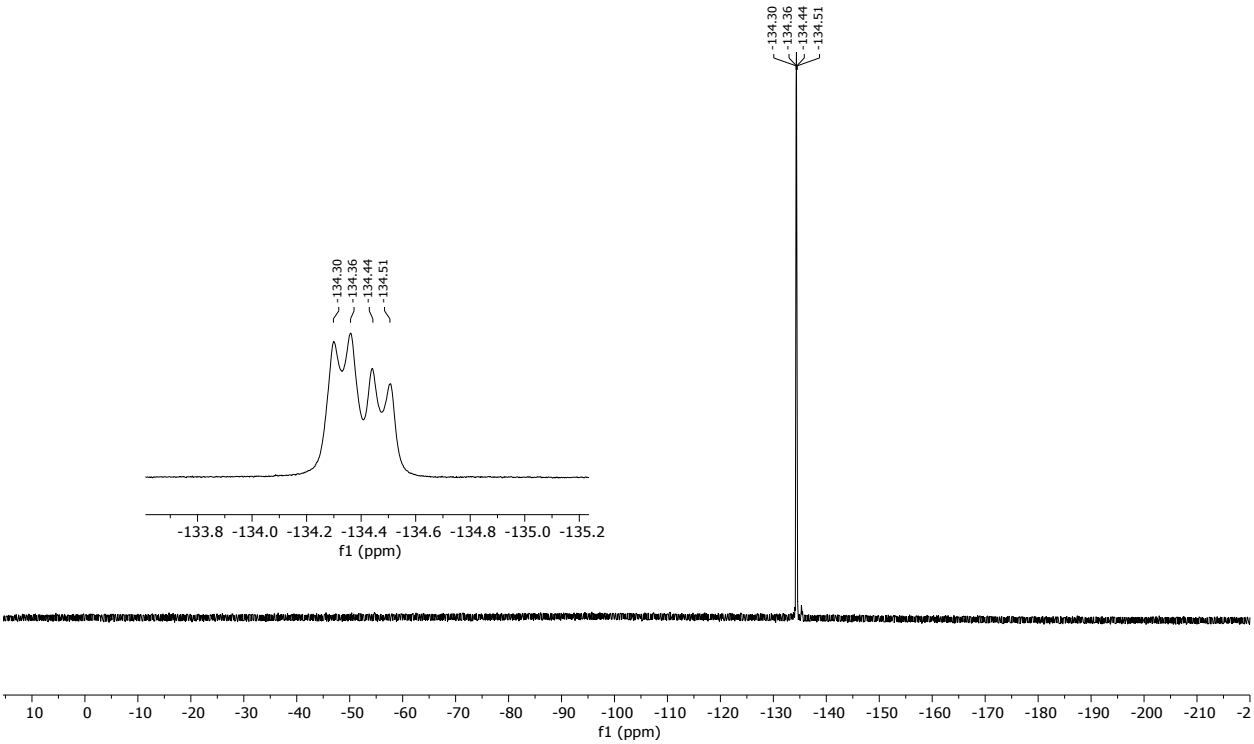


Figure S14. ^{19}F NMR of red-HyP analogue **1** (DMSO-d_6).

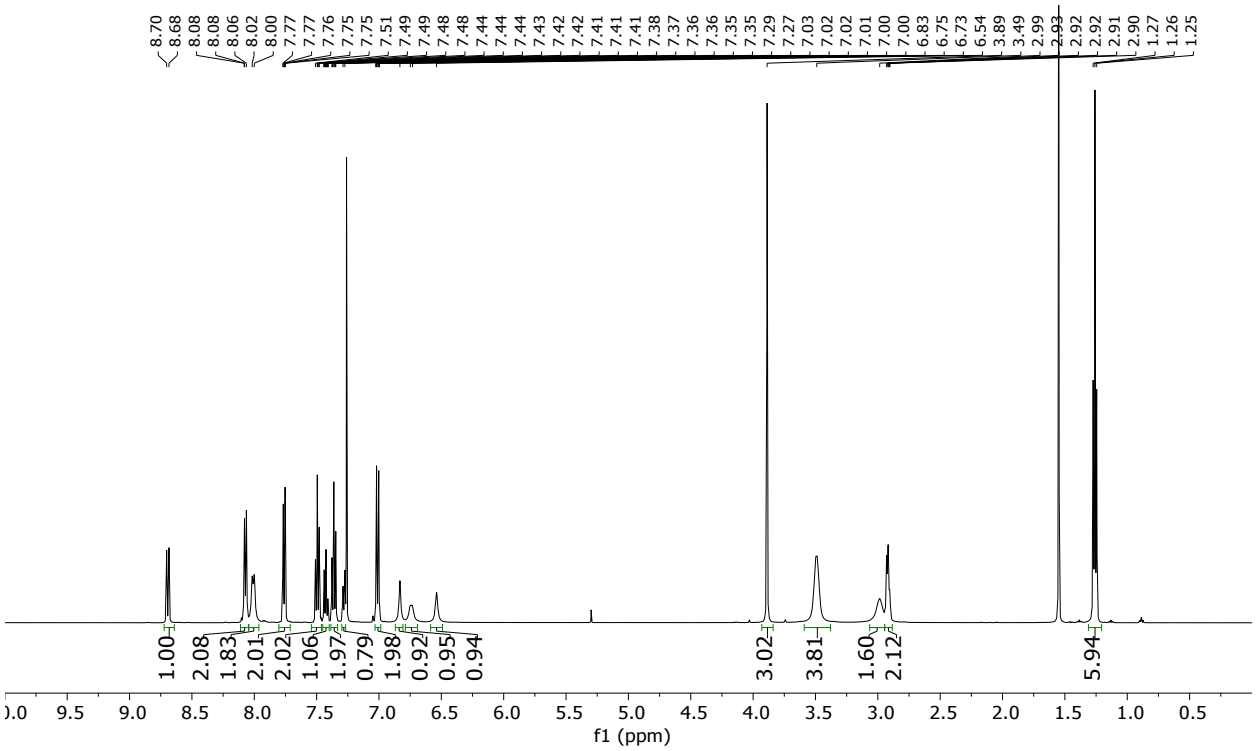


Figure S15. ^{1}H NMR of red-HyP analogue **2** ($CDCl_3$).

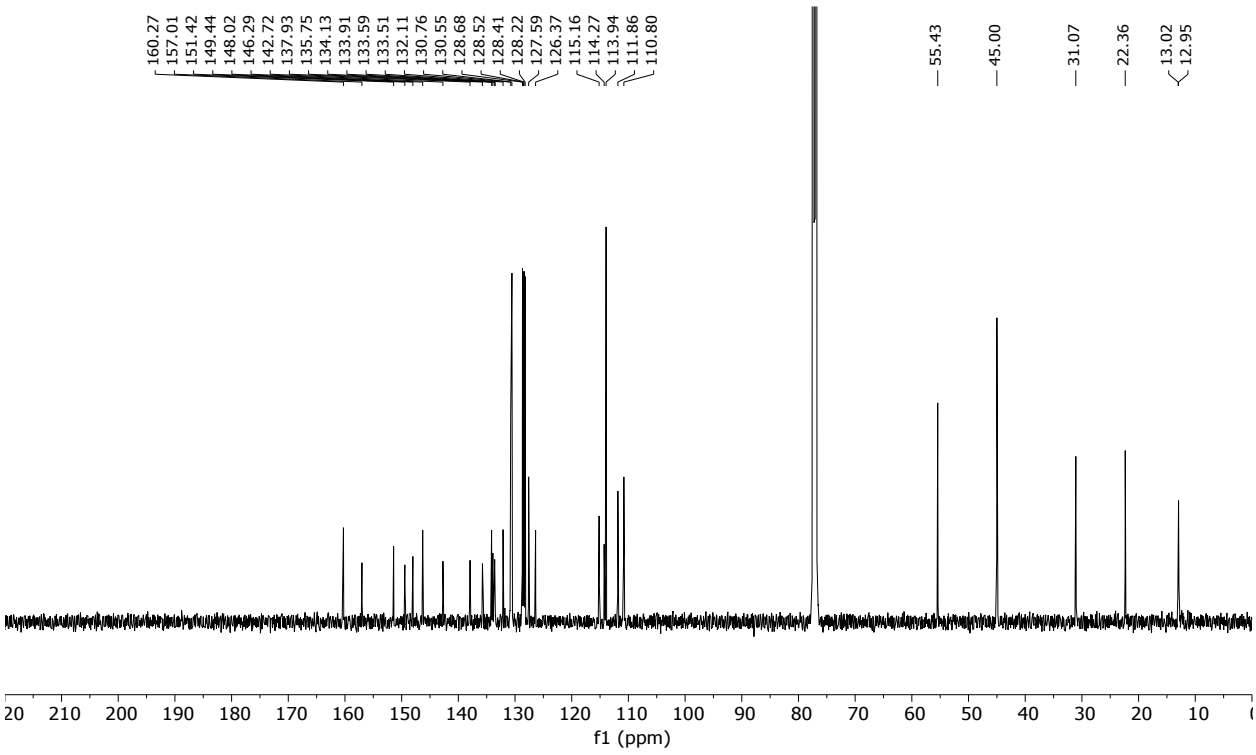


Figure S16. ^{13}C NMR of red-HyP analogue **2** ($CDCl_3$).

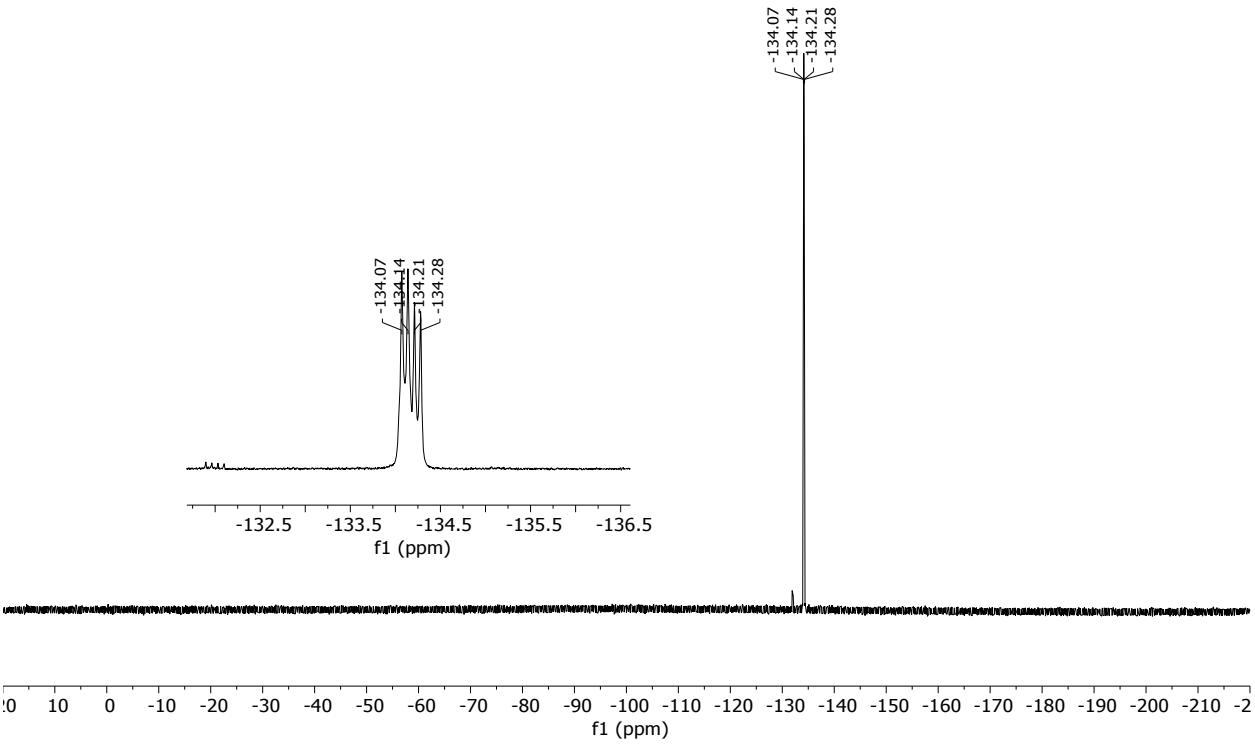


Figure S17. ^{19}F NMR of red-HyP analogue **2** ($CDCl_3$).

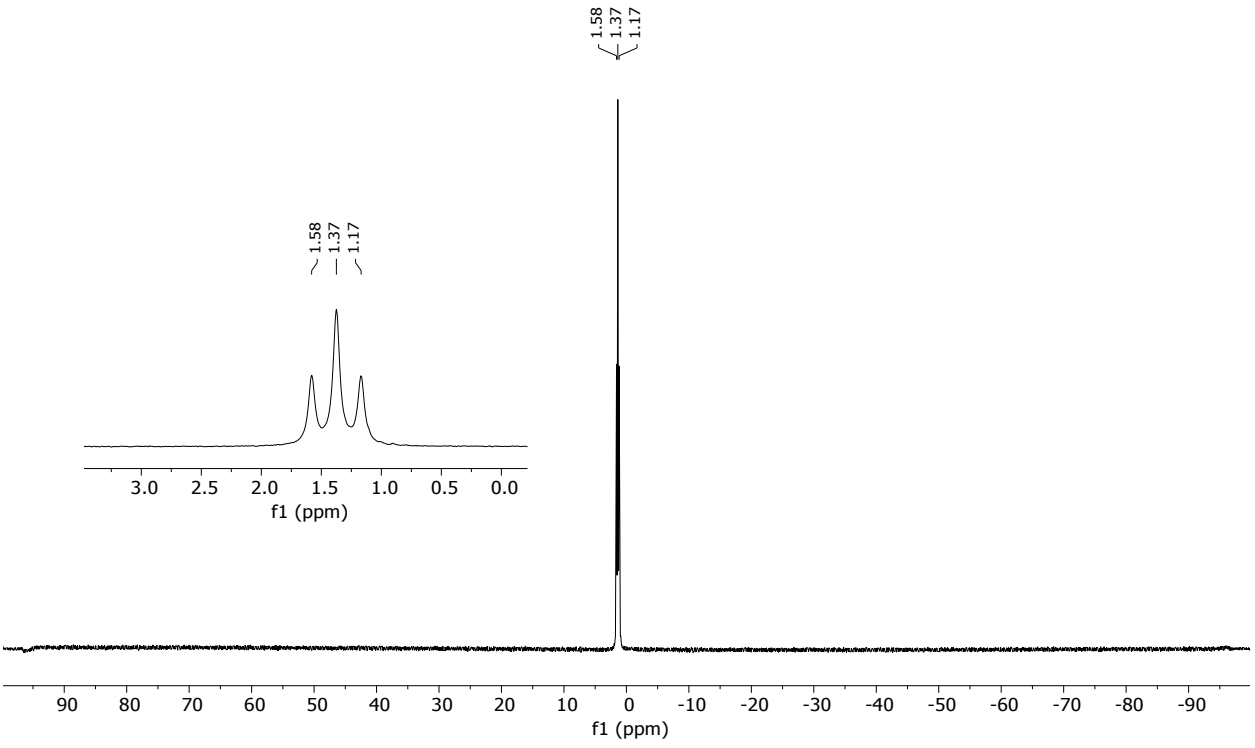


Figure S18. ^{11}B NMR of red-HyP analogue **2** ($CDCl_3$).

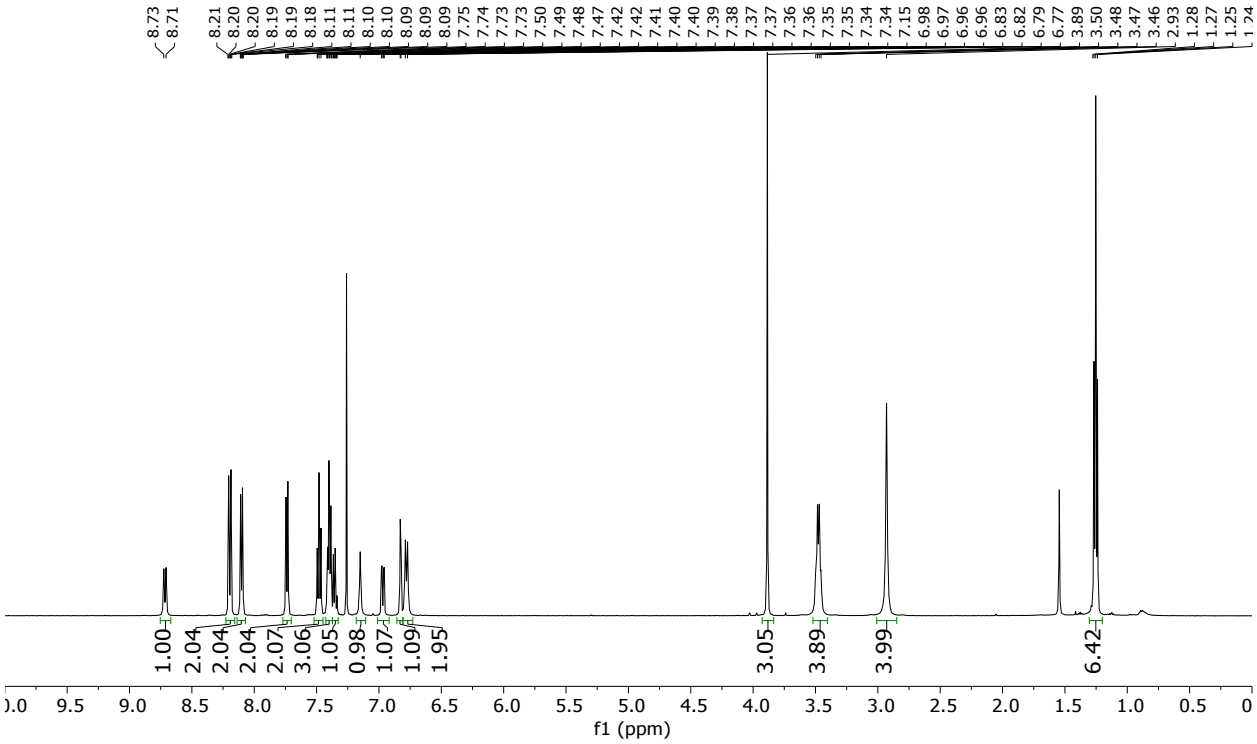


Figure S19. ^{1}H NMR of red-HyP analogue **3** ($CDCl_3$).

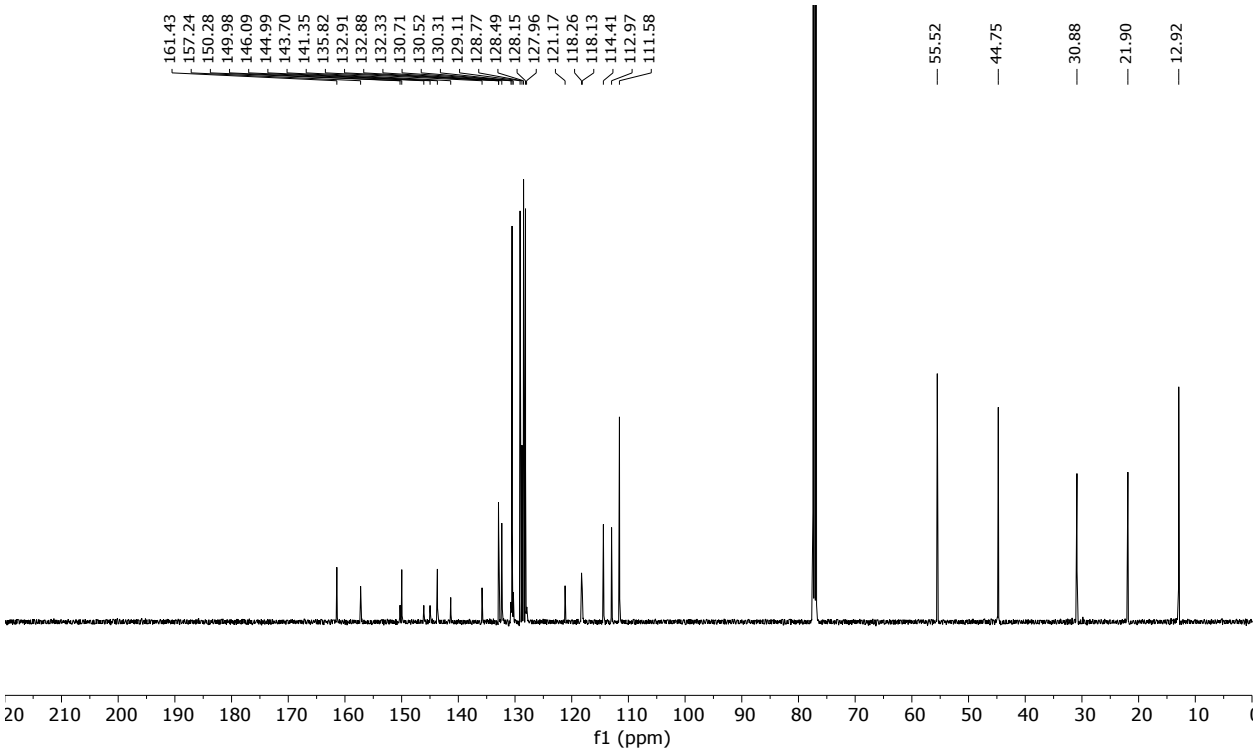


Figure S20. ^{13}C NMR of red-HyP analogue **3** ($CDCl_3$).

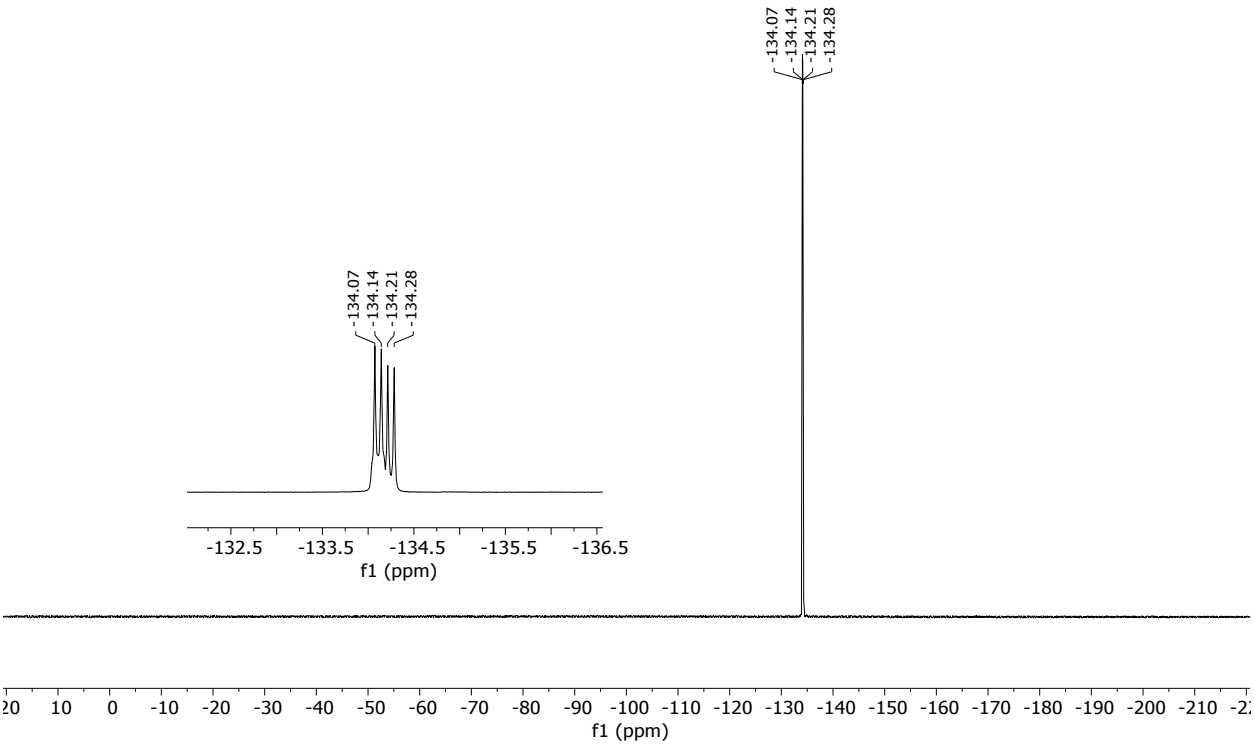


Figure S21. ^{19}F NMR of red-HyP analogue **3** ($CDCl_3$).

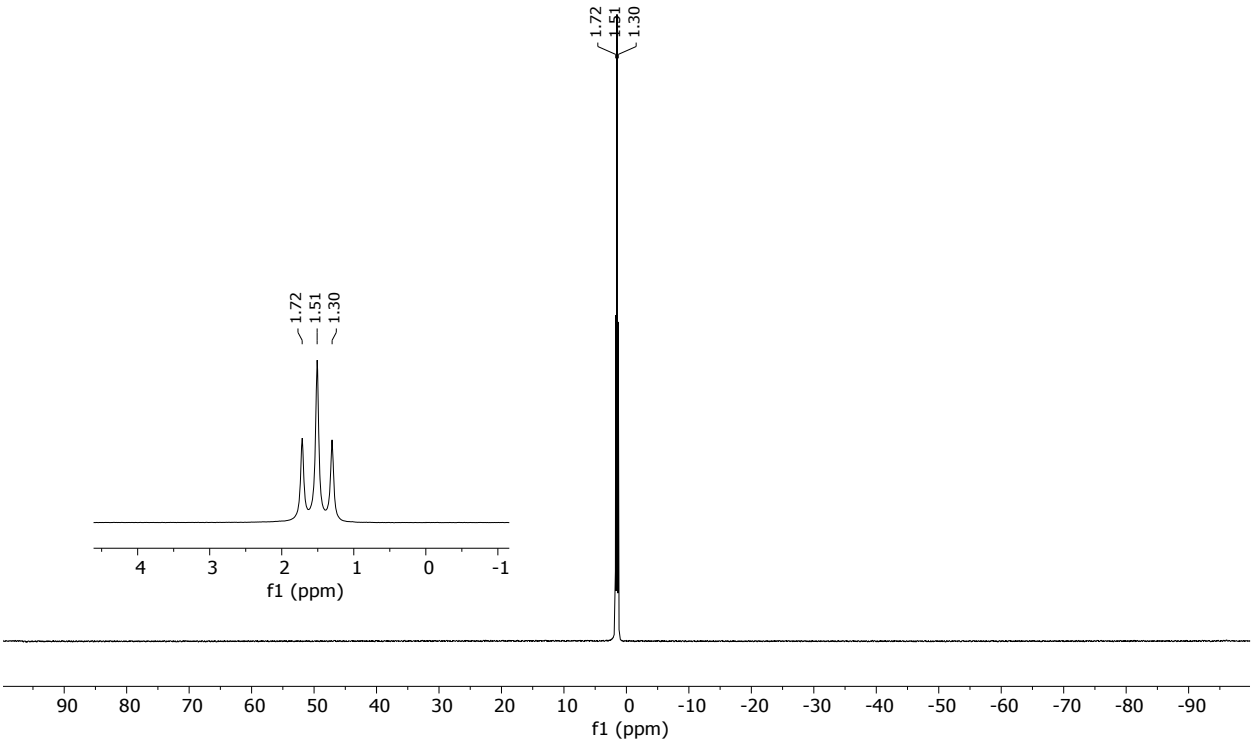


Figure S22. ^{11}B NMR of red-HyP analogue **3** ($CDCl_3$).

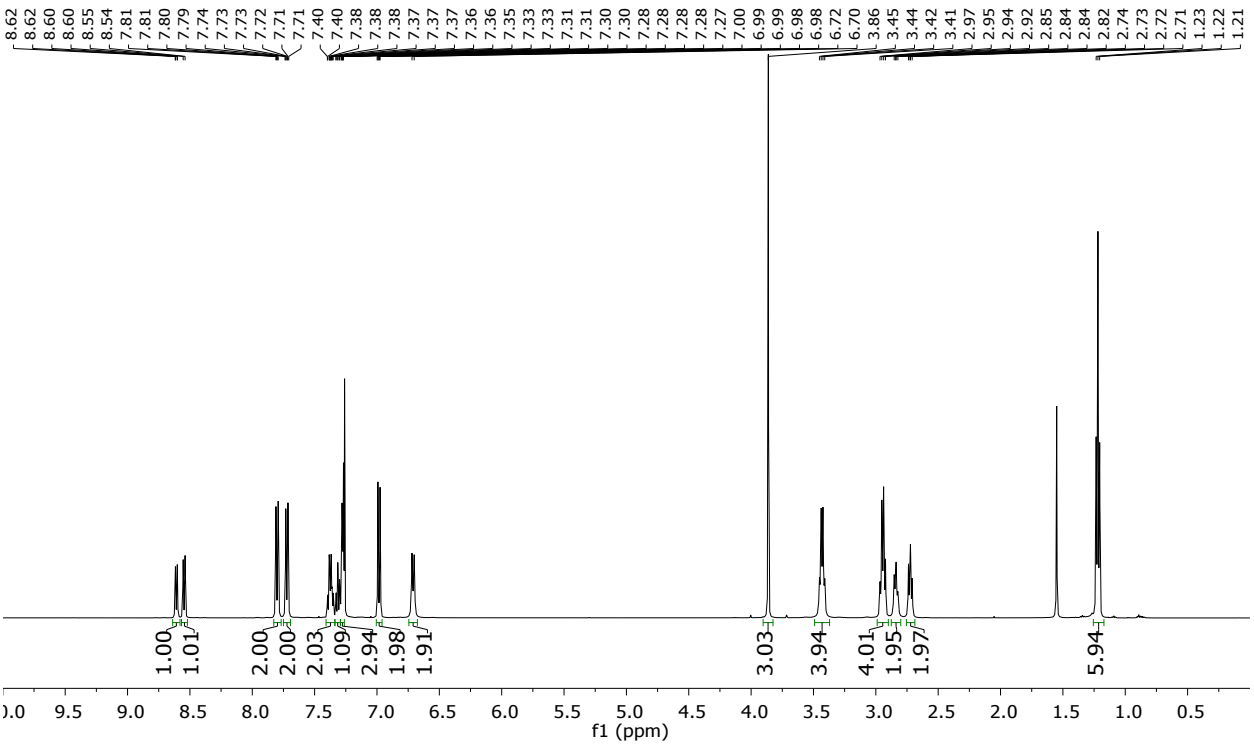


Figure S23. ^{1}H NMR of red-HyP analogue **4** ($CDCl_3$).

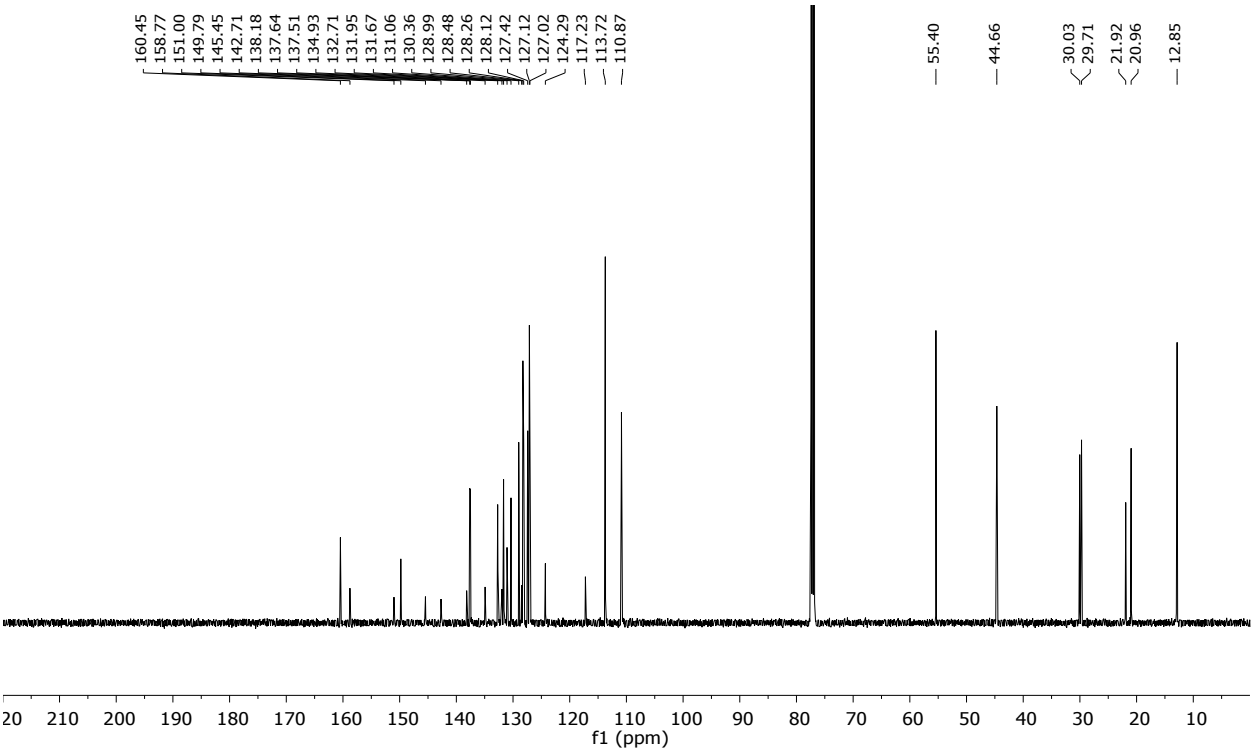


Figure S24. ^{13}C NMR of red-HyP analogue **4** ($CDCl_3$).

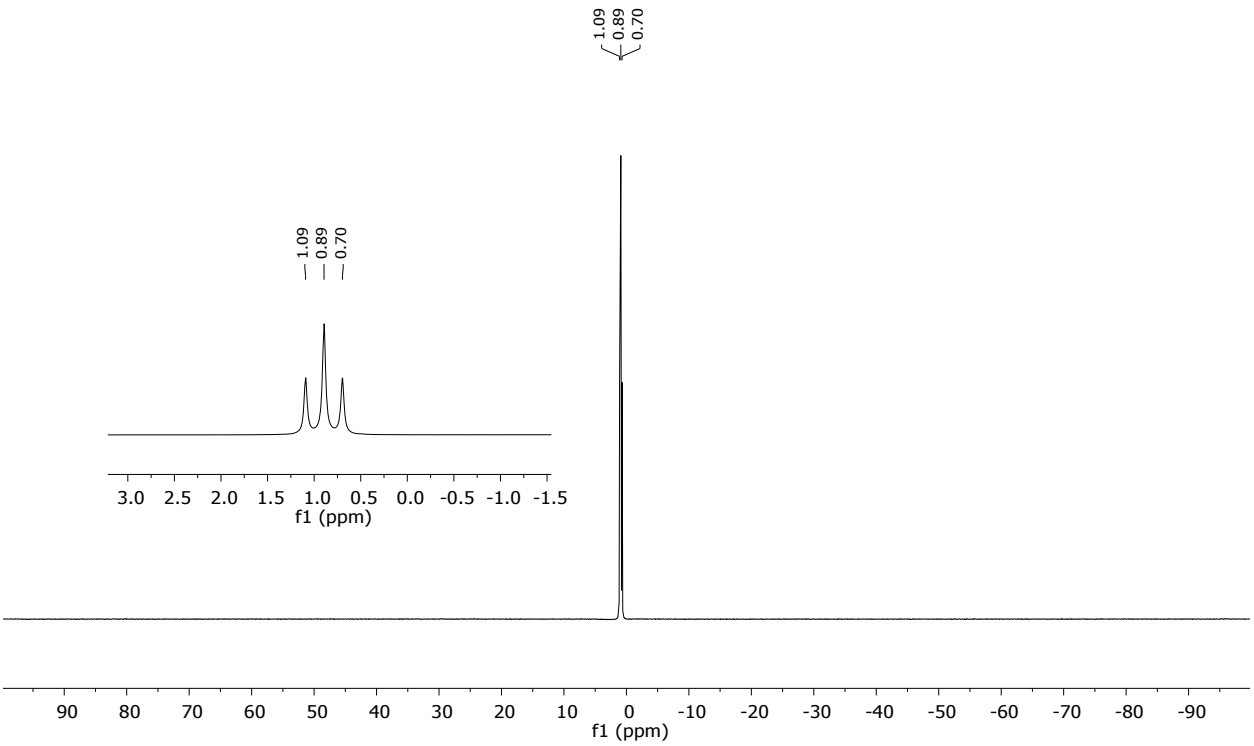


Figure S25. ^{11}B NMR of red-HyP analogue **4** ($CDCl_3$).

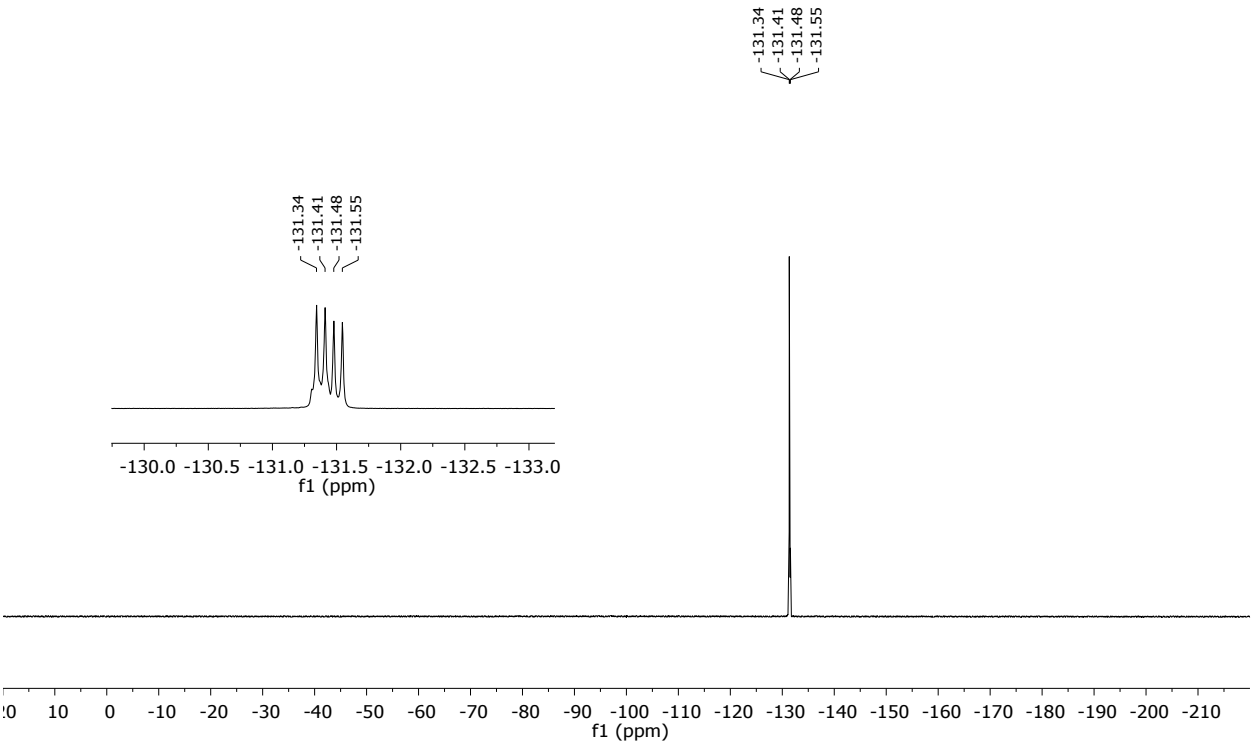


Figure S26. ^{19}F NMR of red-HyP analogue **4** ($CDCl_3$).

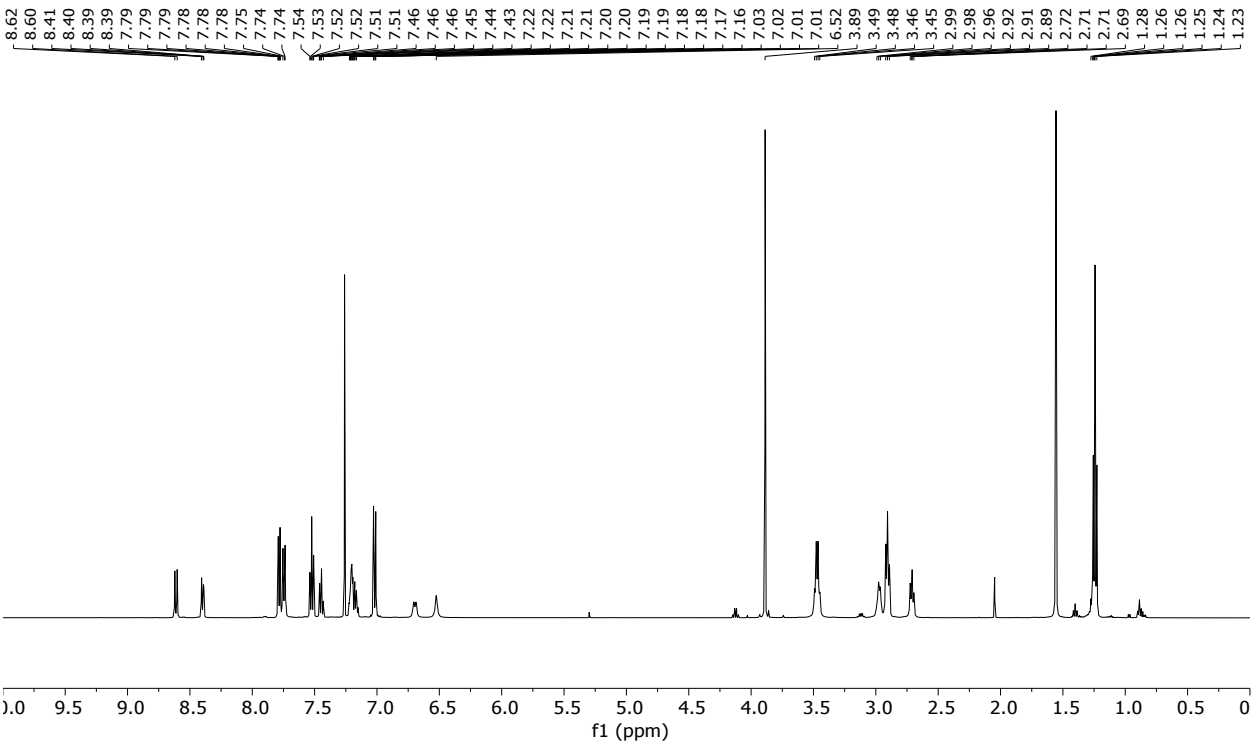


Figure S27. ^{1}H NMR of red-HyP analogue **5** ($CDCl_3$).

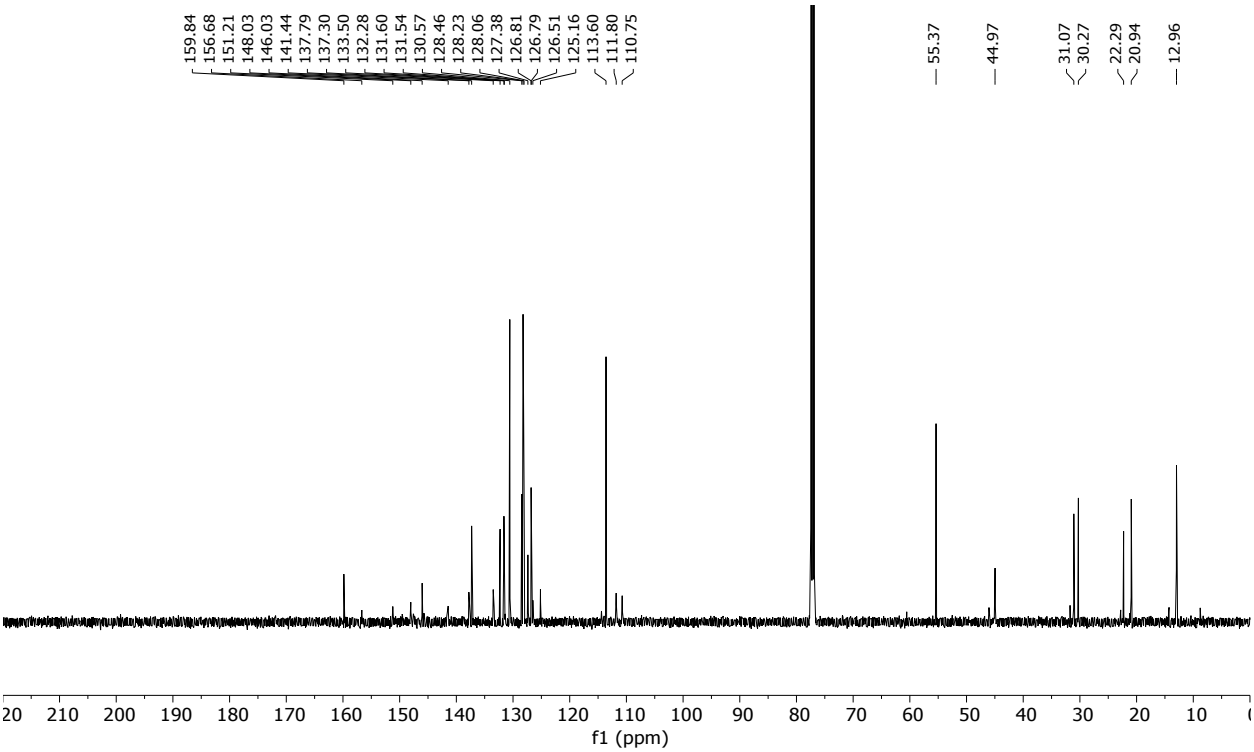


Figure S28. ^{13}C NMR of red-HyP analogue **5** ($CDCl_3$).

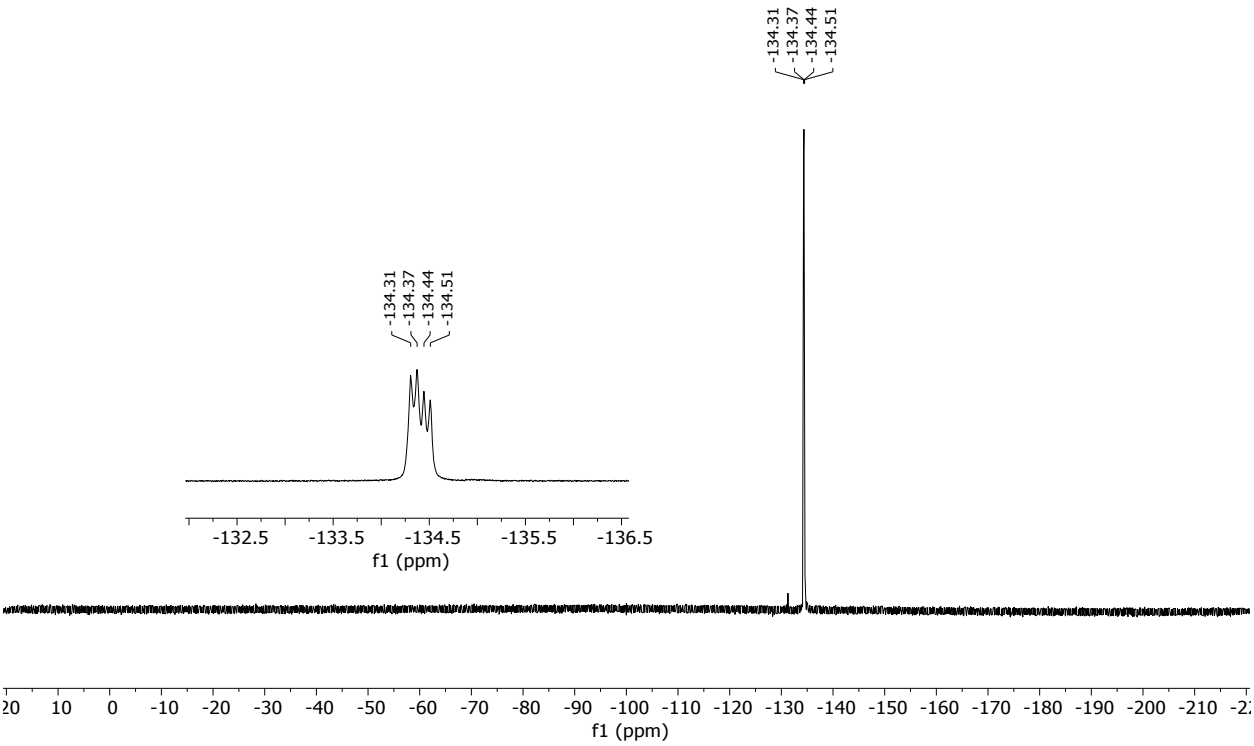


Figure S29. ^{19}F NMR of red-HyP analogue **5** ($CDCl_3$).

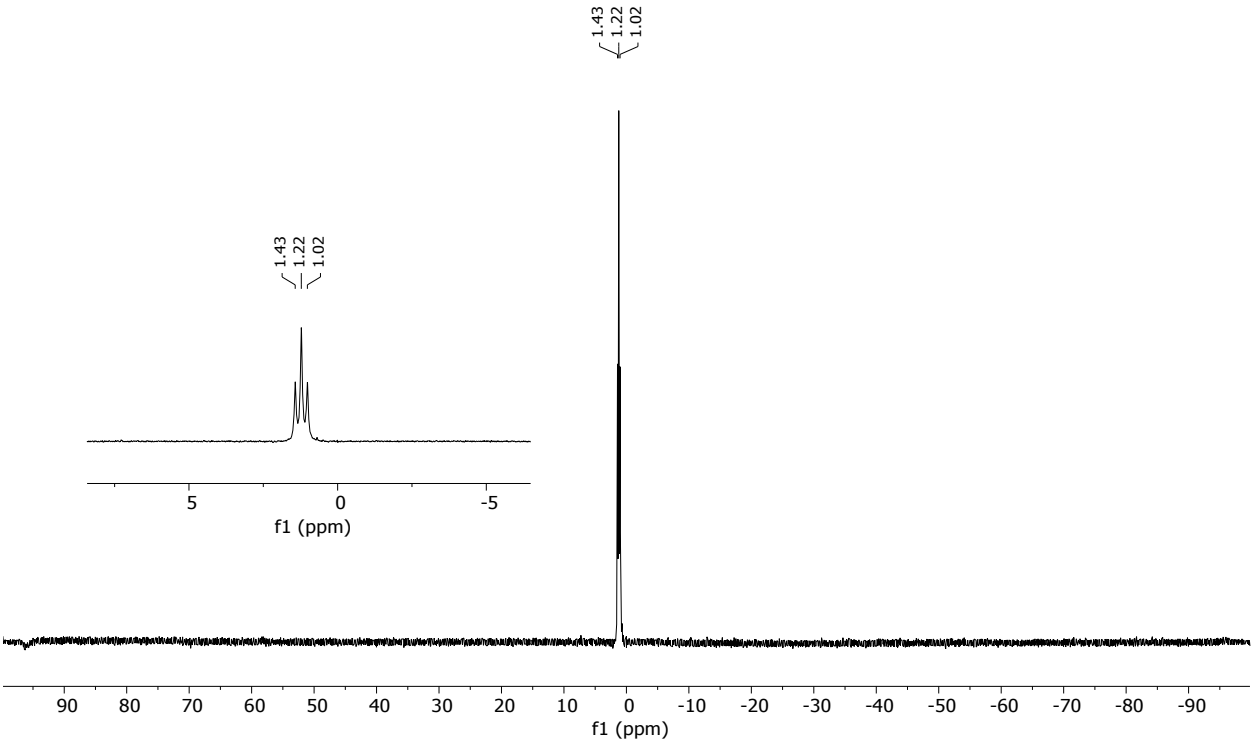


Figure S30. ^{11}B NMR of red-HyP analogue **5** ($CDCl_3$).

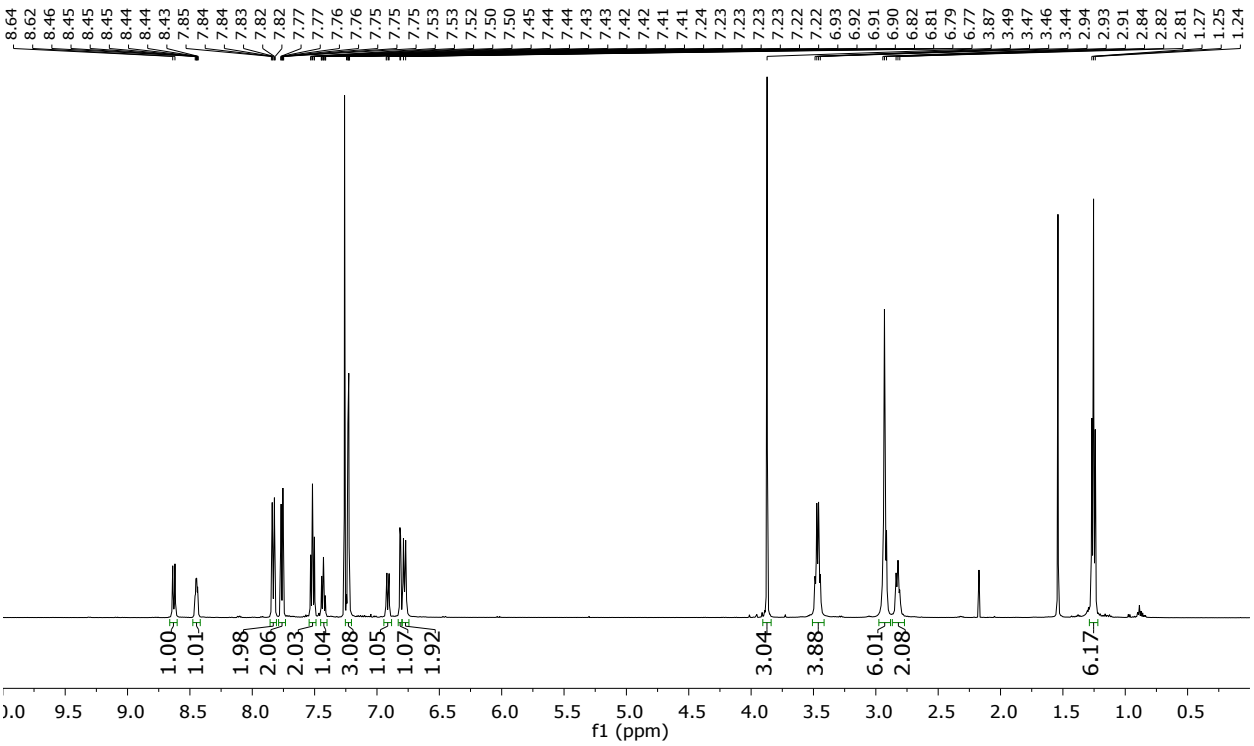


Figure S31. ^{1}H NMR of red-HyP analogue **6** ($CDCl_3$).

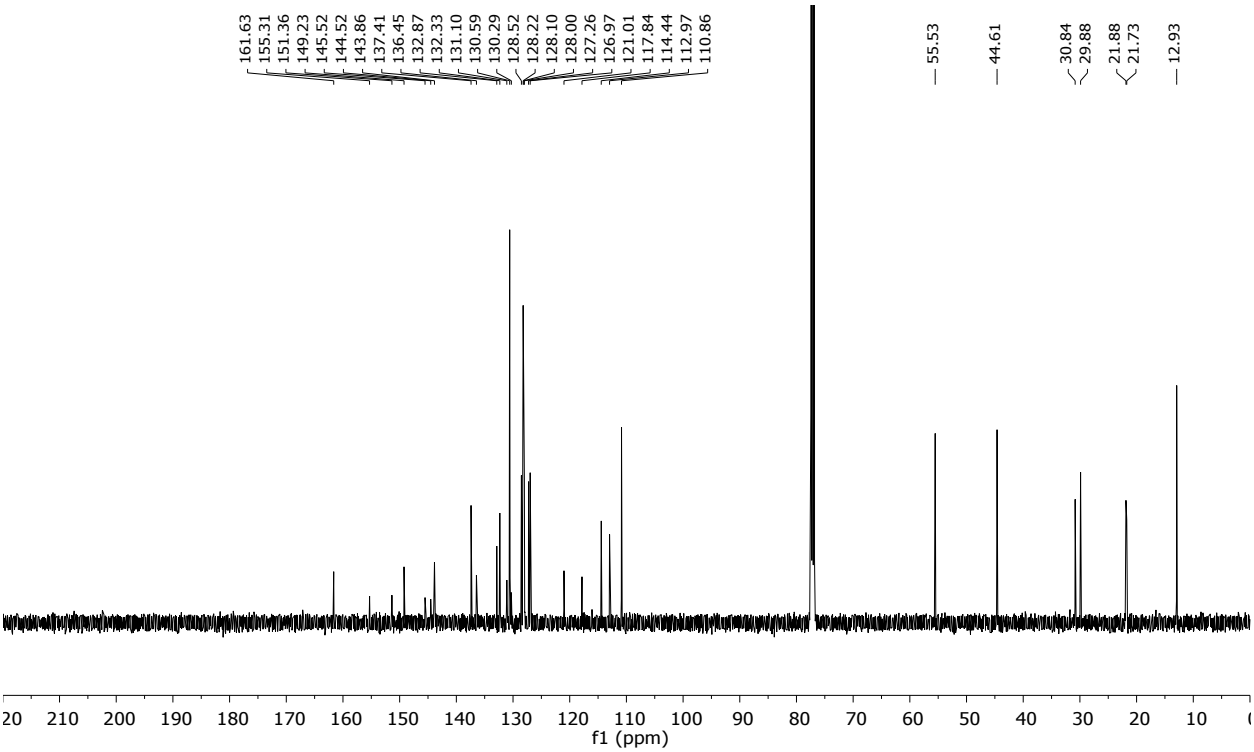


Figure S32. ^{13}C NMR of red-HyP analogue **6** ($CDCl_3$).

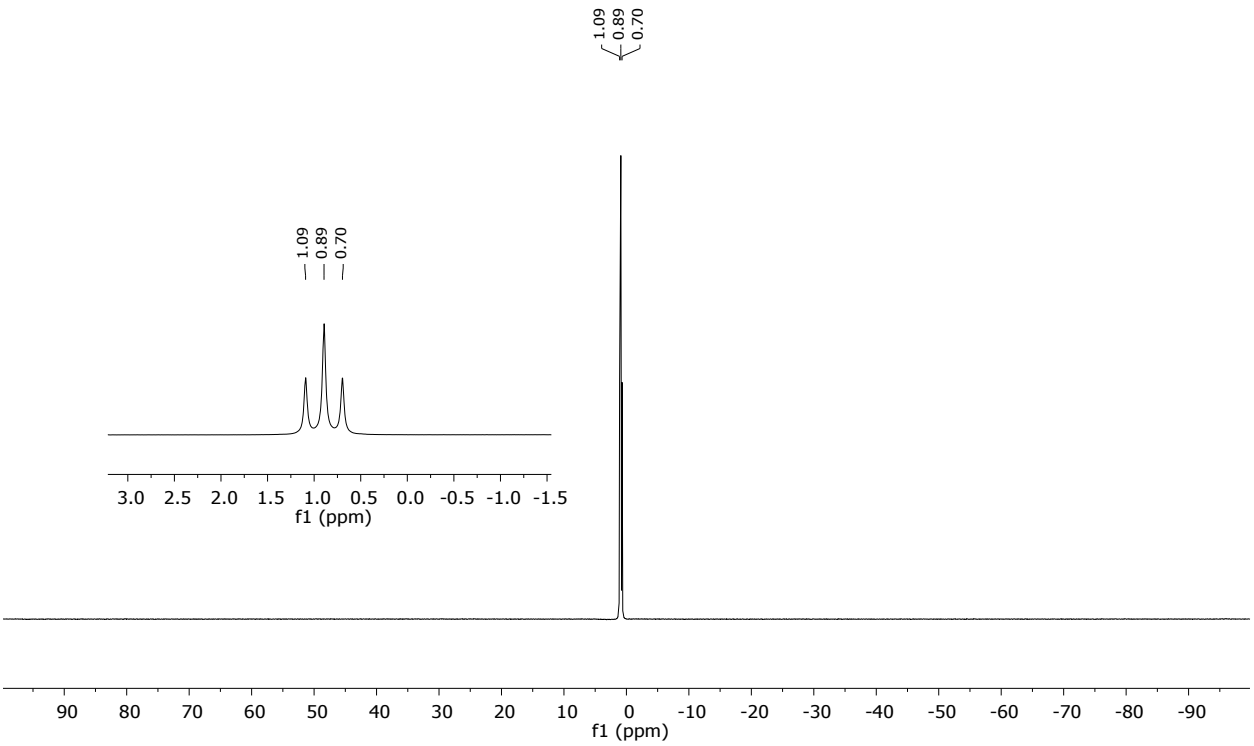


Figure S33. ^{11}B NMR of red-HyP analogue **6** ($CDCl_3$).

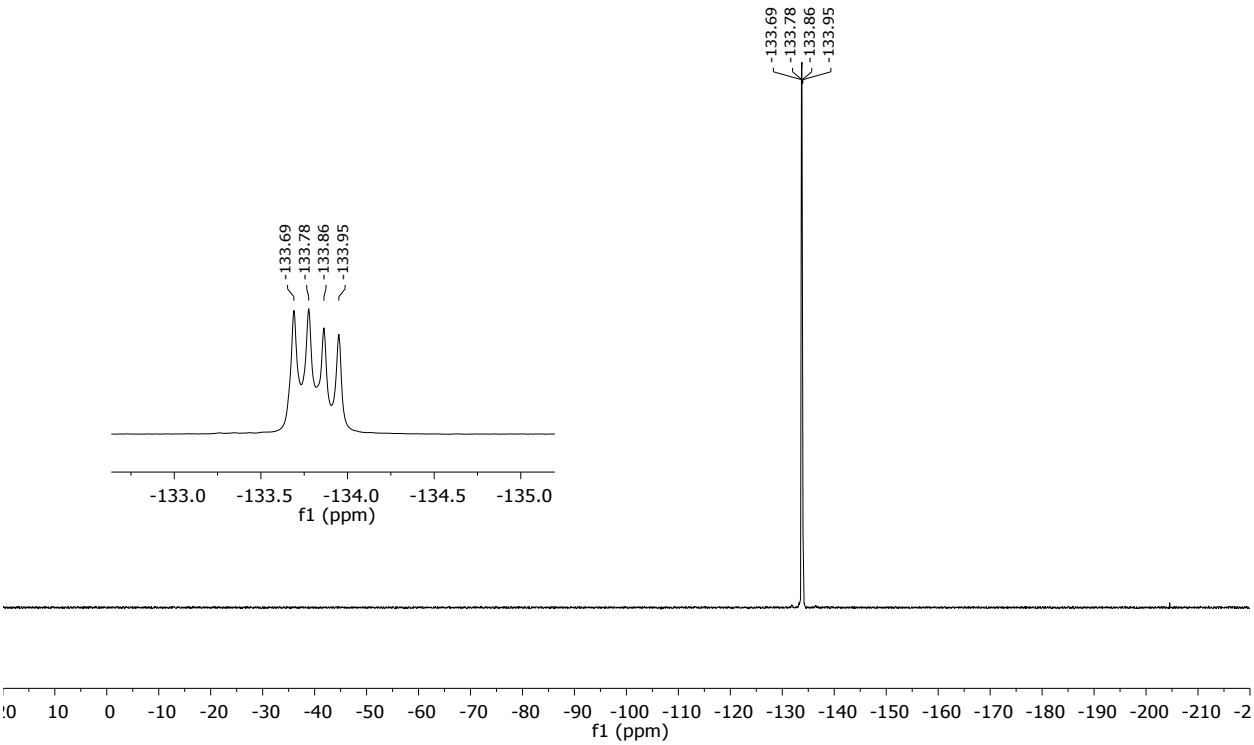


Figure S34. ^{19}F NMR of red-HyP analogue **6** ($CDCl_3$).

NMR Spectra of Probes and Products:

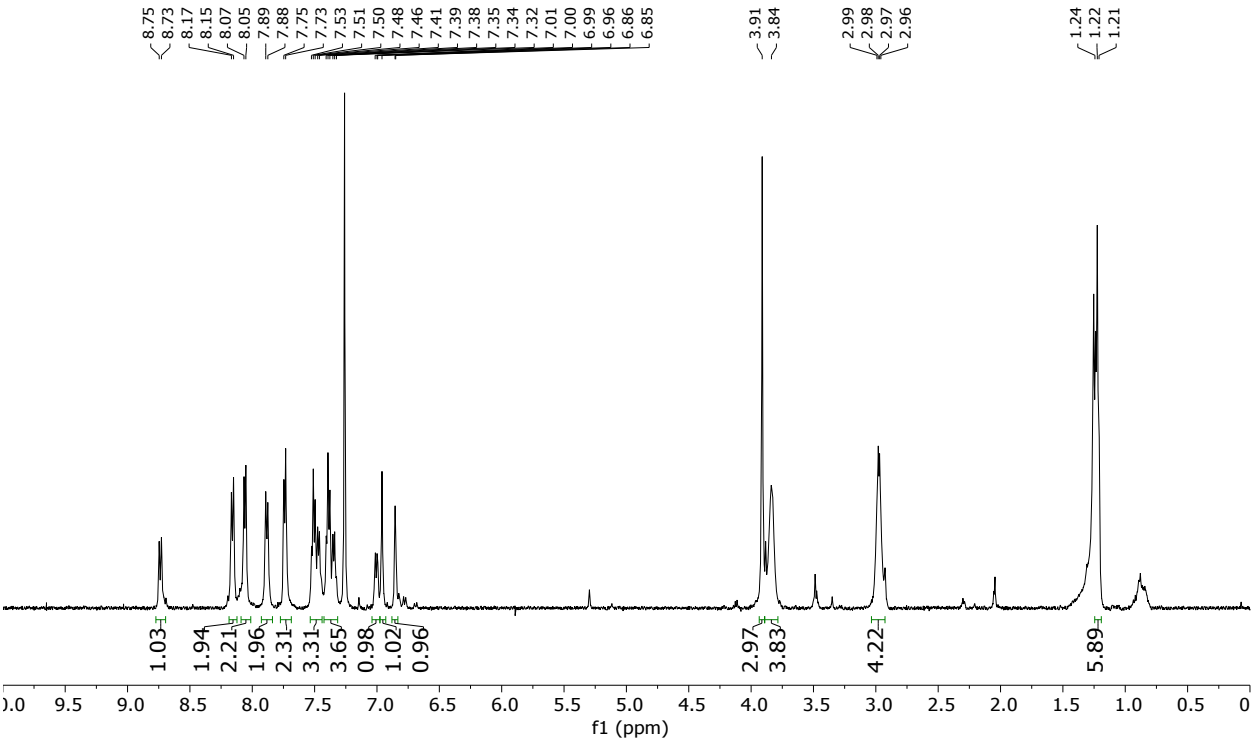


Figure S35. ^{1}H NMR of **CRaB-HyP** ($CDCl_3$).

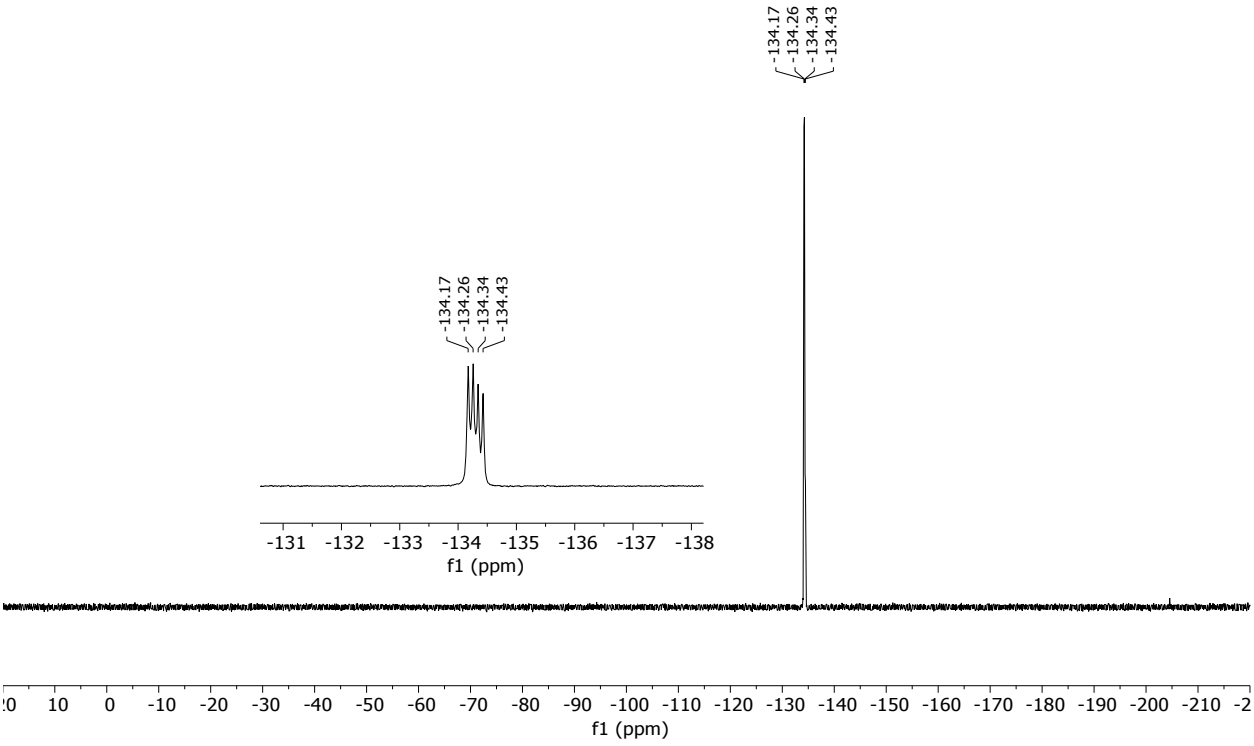


Figure S36. ^{19}F NMR of **CRaB-HyP** (CD_2Cl_2).

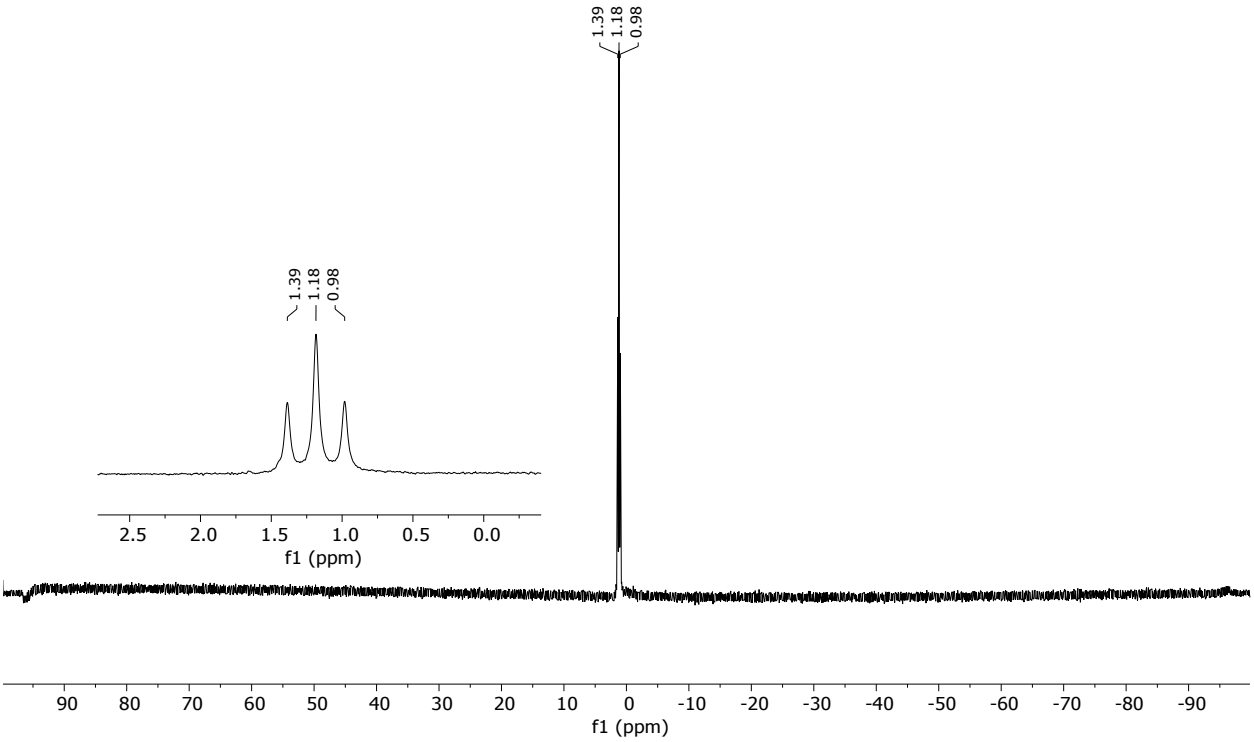


Figure S37. ^{11}B NMR of **CRaB-HyP** (CD_2Cl_2).

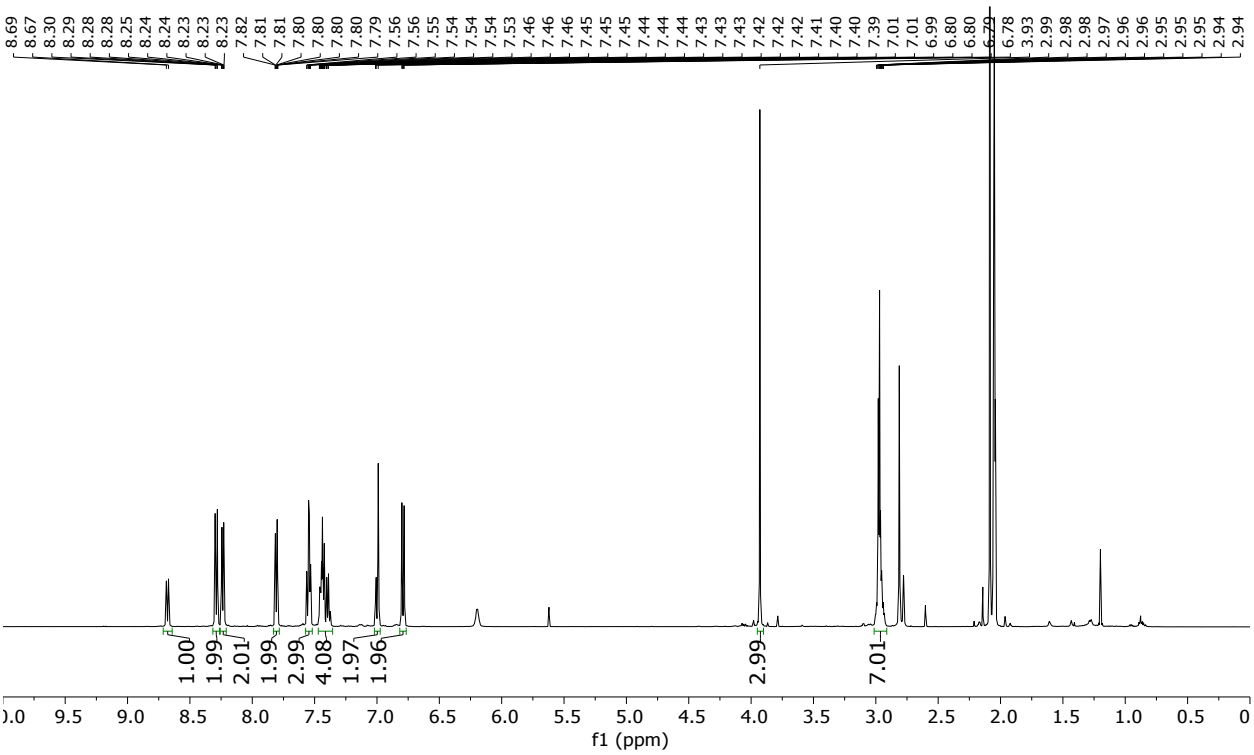


Figure S38. ^{1}H NMR of **r-CRaB-NOD** (Acetone-d_6).

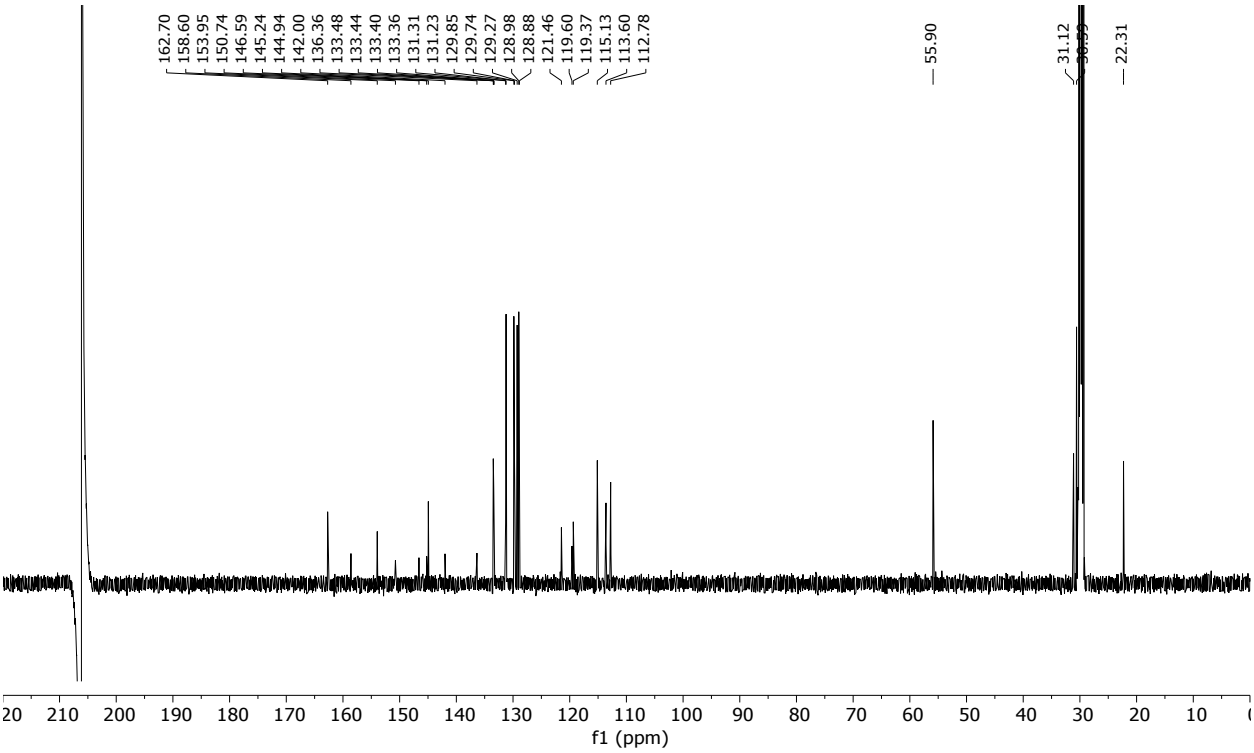


Figure S39. ^{13}C NMR of **r-CRaB-NOD** (Acetone-d_6).

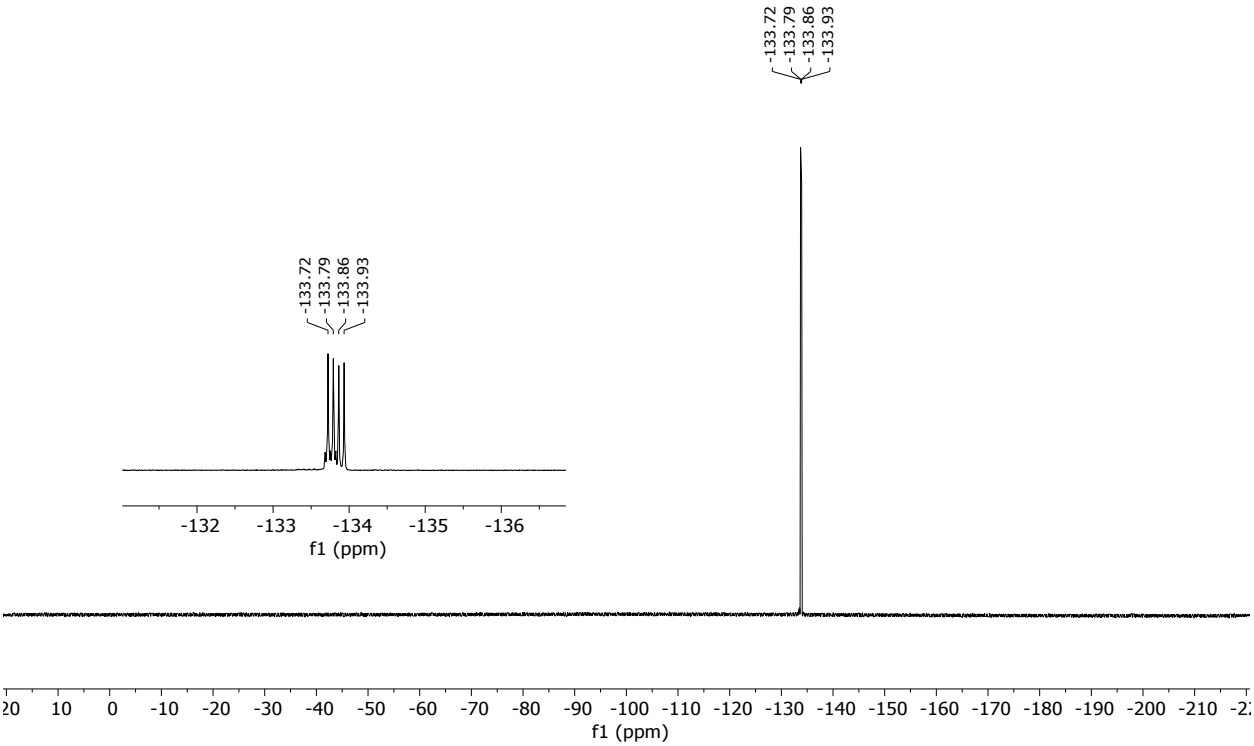


Figure S40. ^{19}F NMR of **r-CRaB-NOD** (Acetone-d_6).

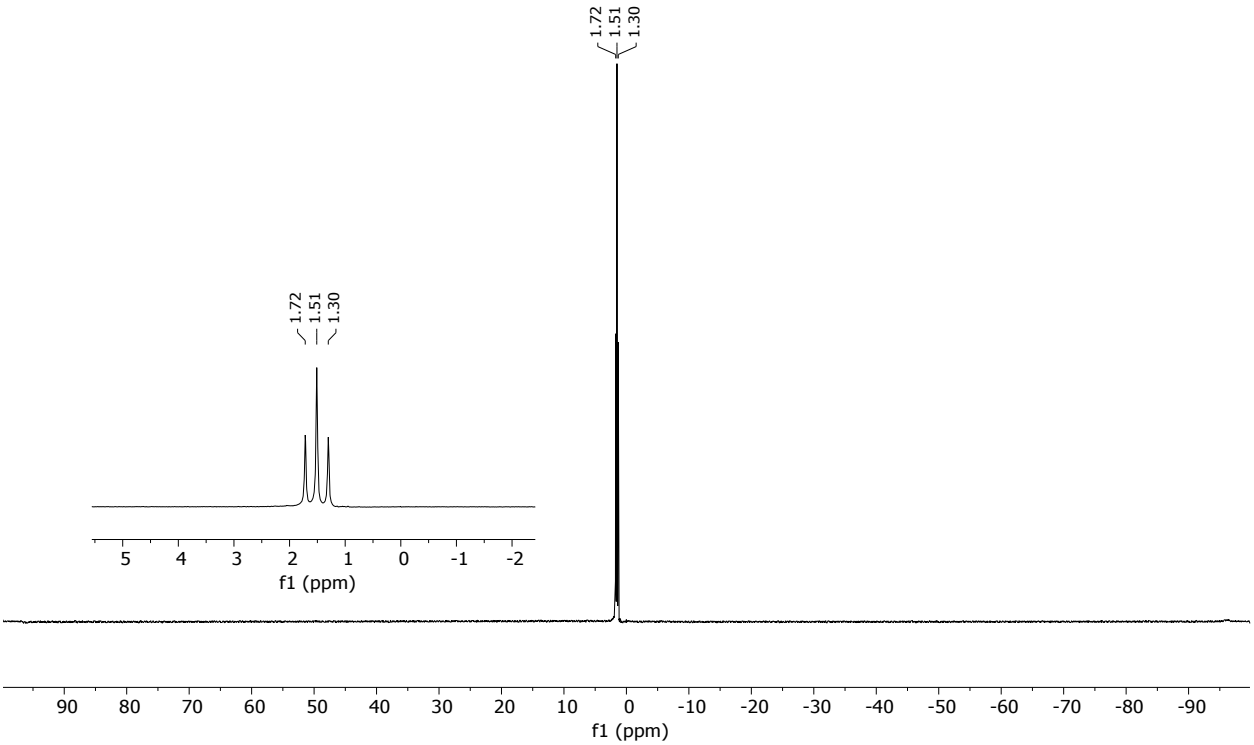


Figure S41. ^{11}B NMR of **r-CRaB-NOD** (Acetone-d_6).

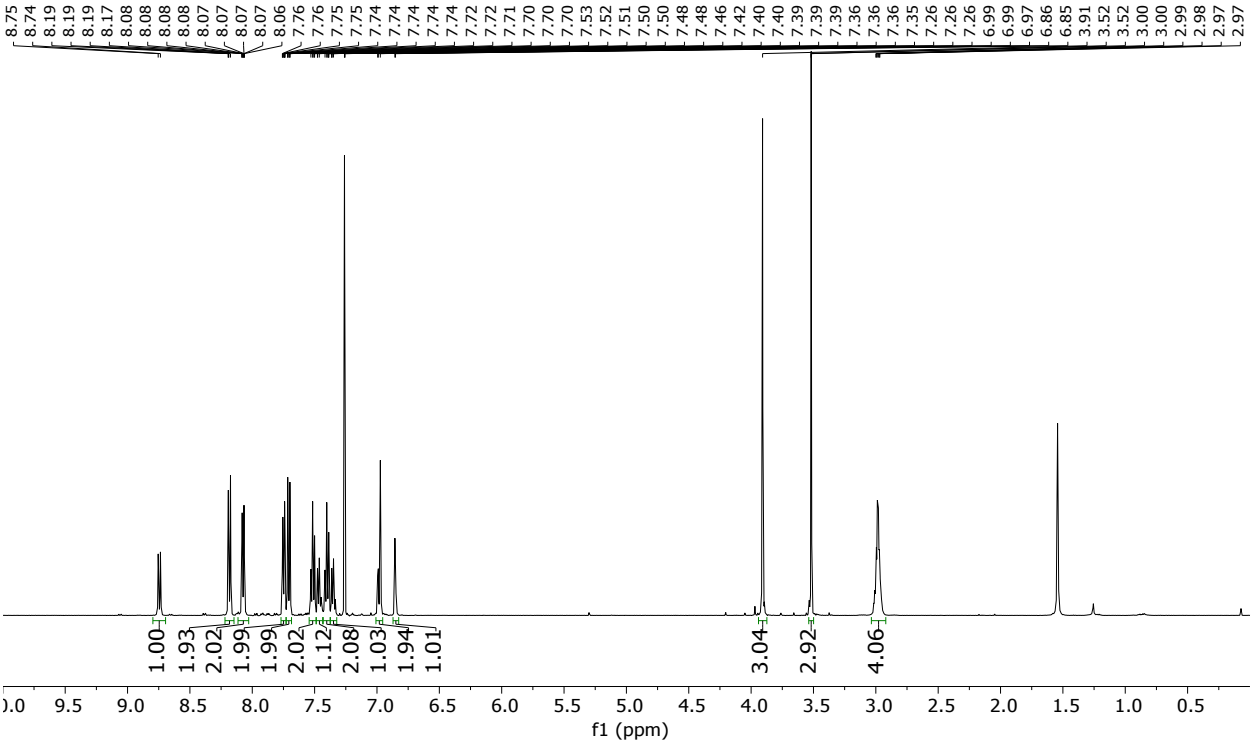


Figure S42. ^{1}H NMR of **CRaB-photoNOD** ($CDCl_3$).

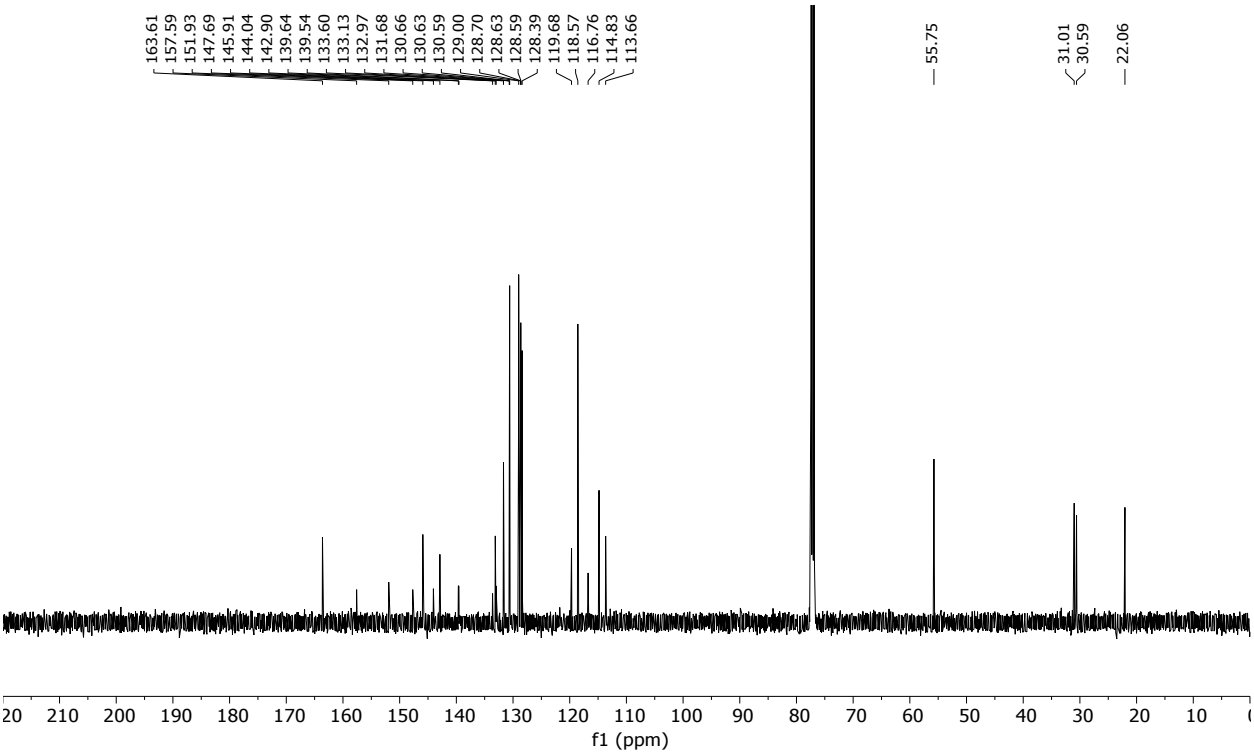


Figure S43. ^{13}C NMR of **CRaB-photoNOD** ($CDCl_3$).

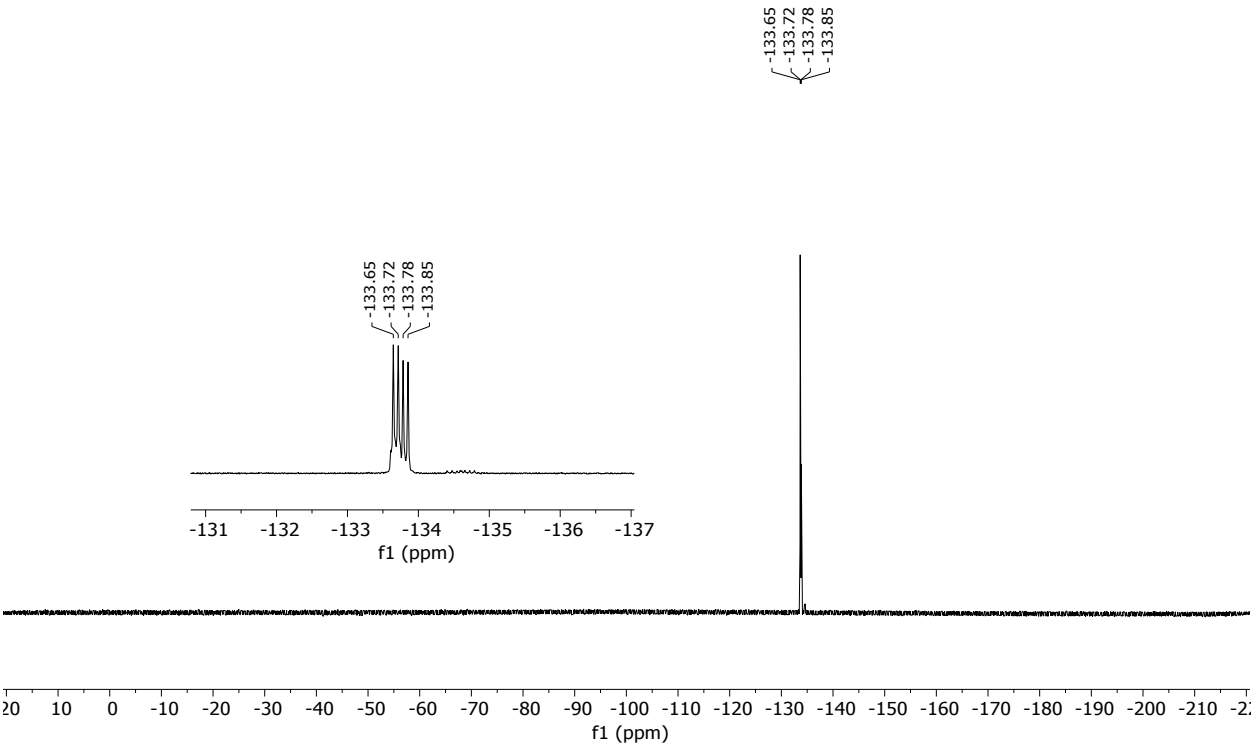


Figure S44. ^{19}F NMR of **CRaB-photoNOD** ($CDCl_3$).

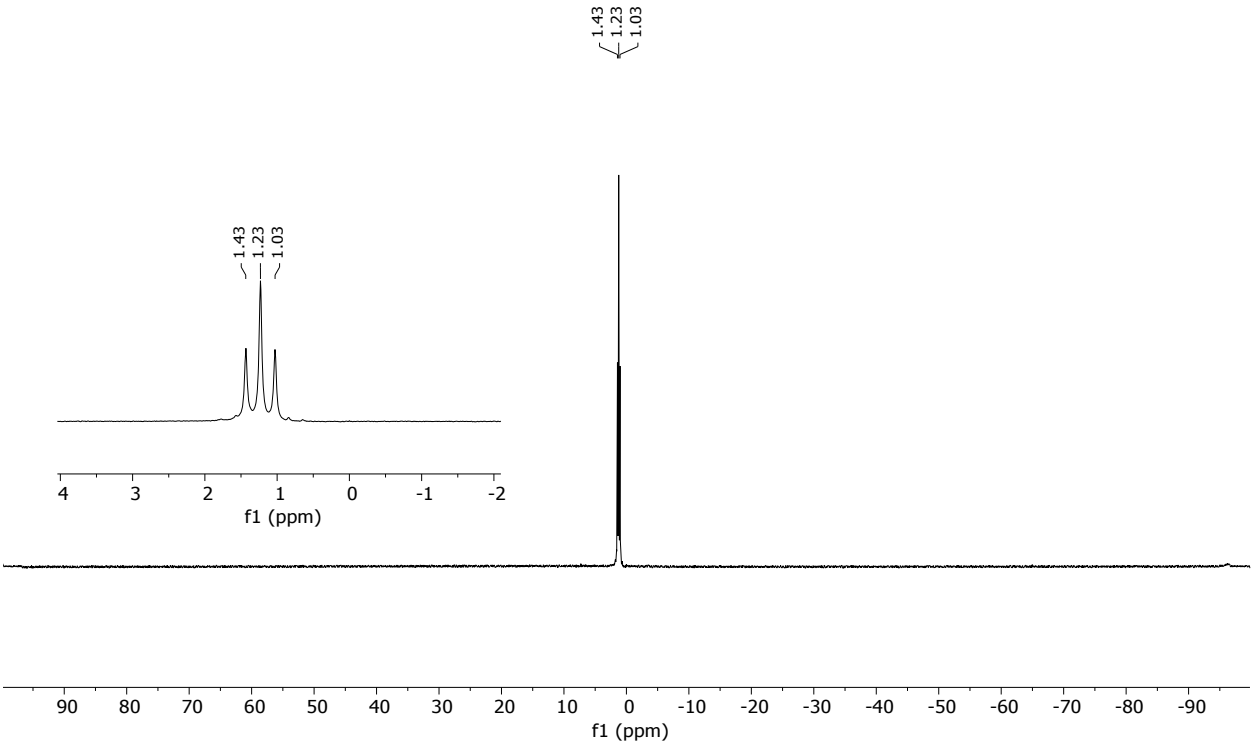


Figure S45. ^{11}B NMR of **CRaB-photoNOD** ($CDCl_3$).

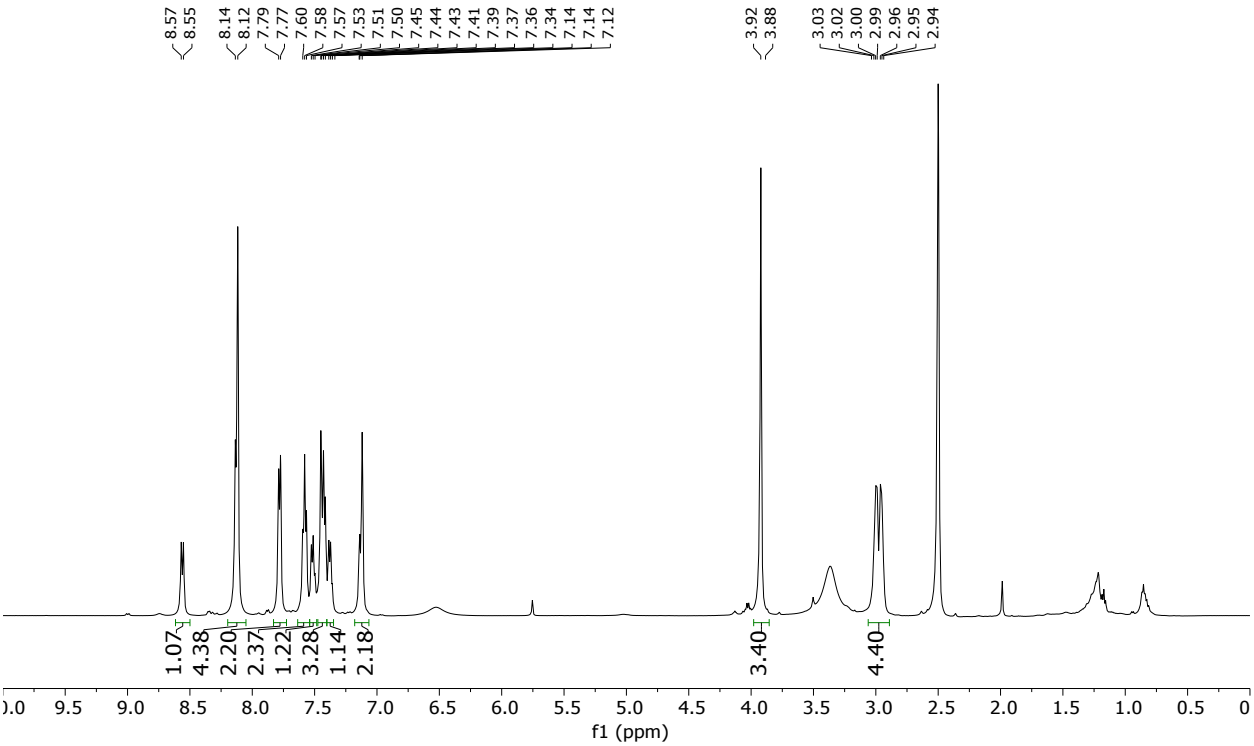


Figure S46. ^{1}H NMR of **t-CRaB-OMe-APC** (DMSO-d_6).

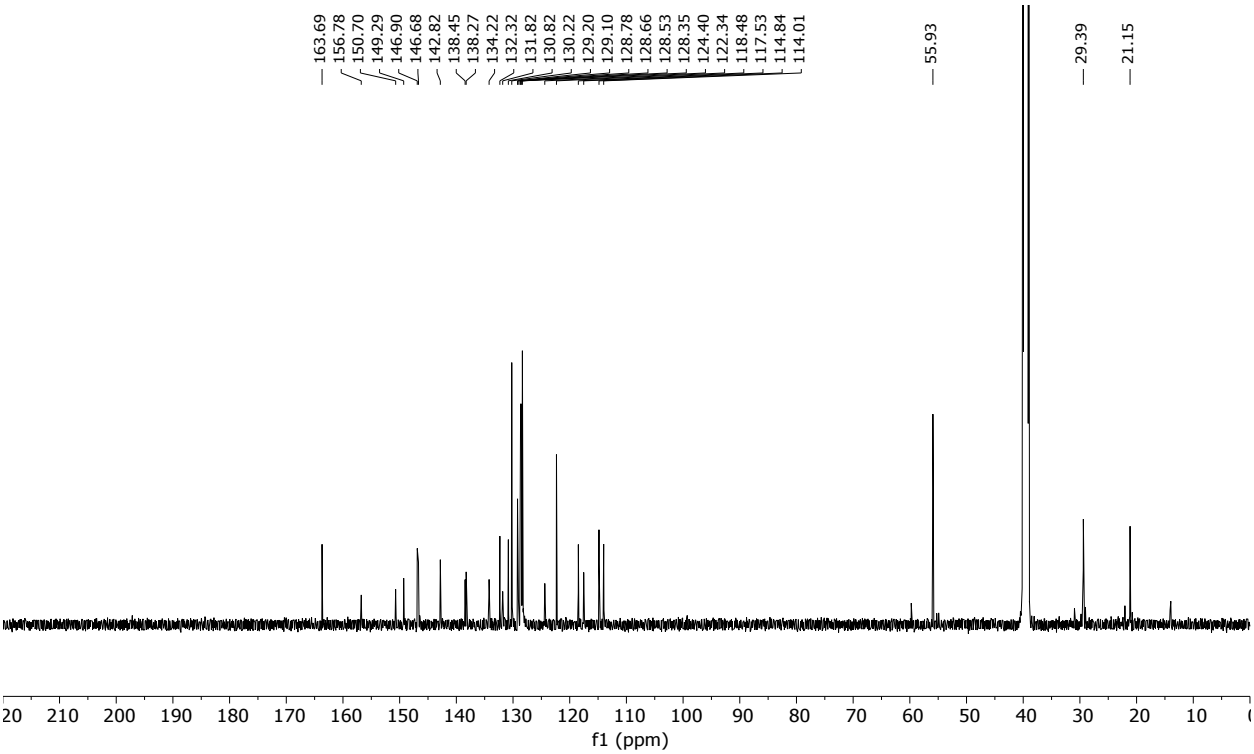


Figure S47. ^{13}C NMR of **t-CRaB-OMe-APC** (DMSO-d_6).

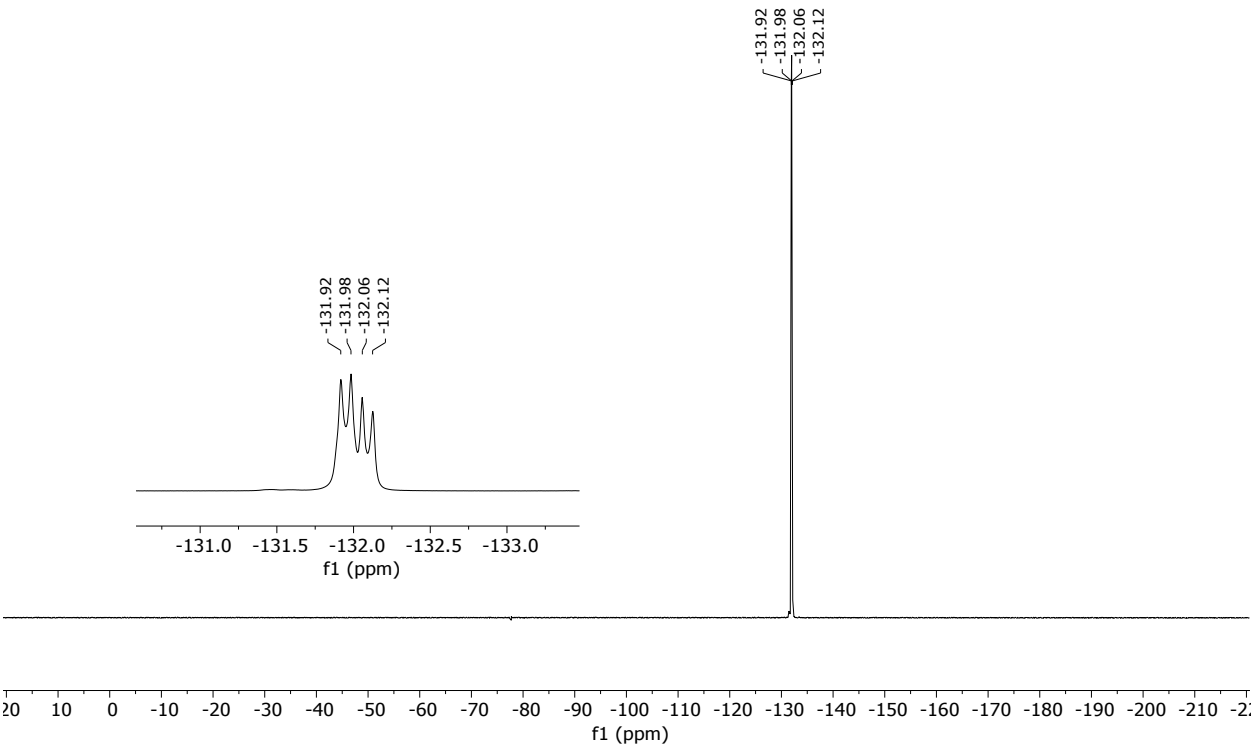


Figure S48. ^{19}F NMR of **t-CRaB-OMe-APC** (DMSO-d_6).

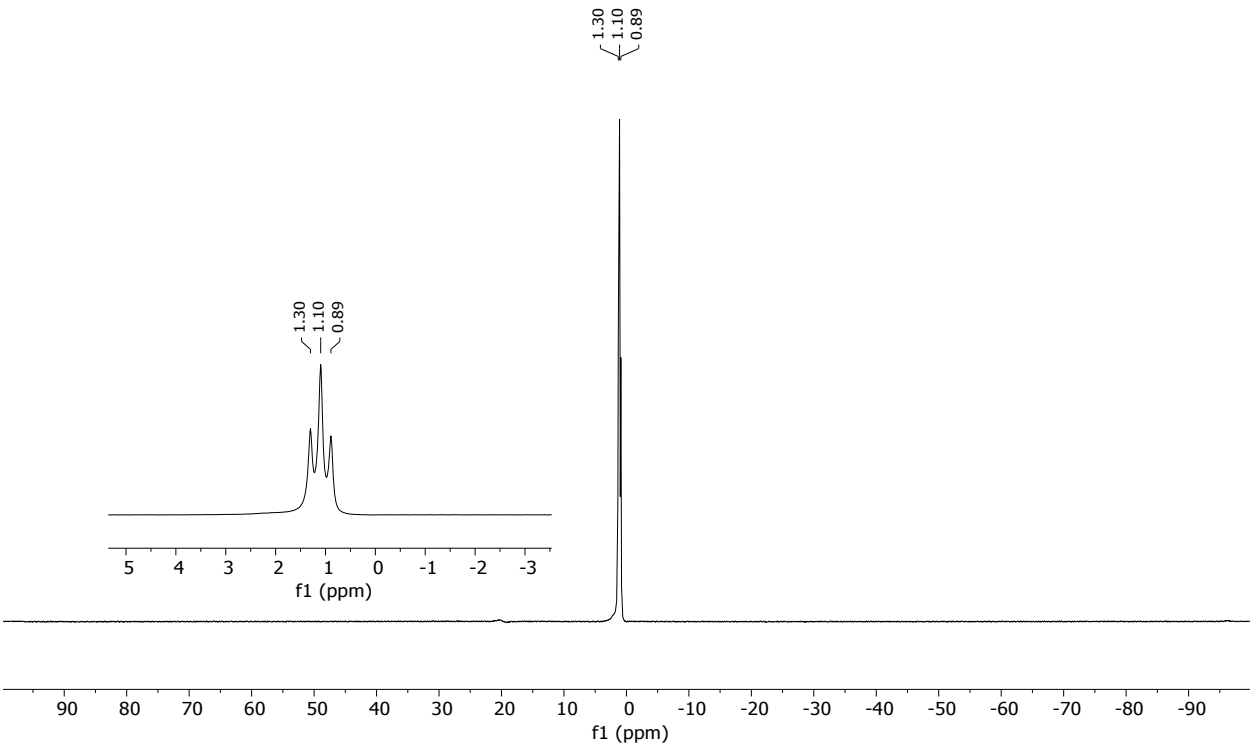


Figure S49. ^{11}B NMR of **t-CRaB-OMe-APC** (DMSO-d_6).

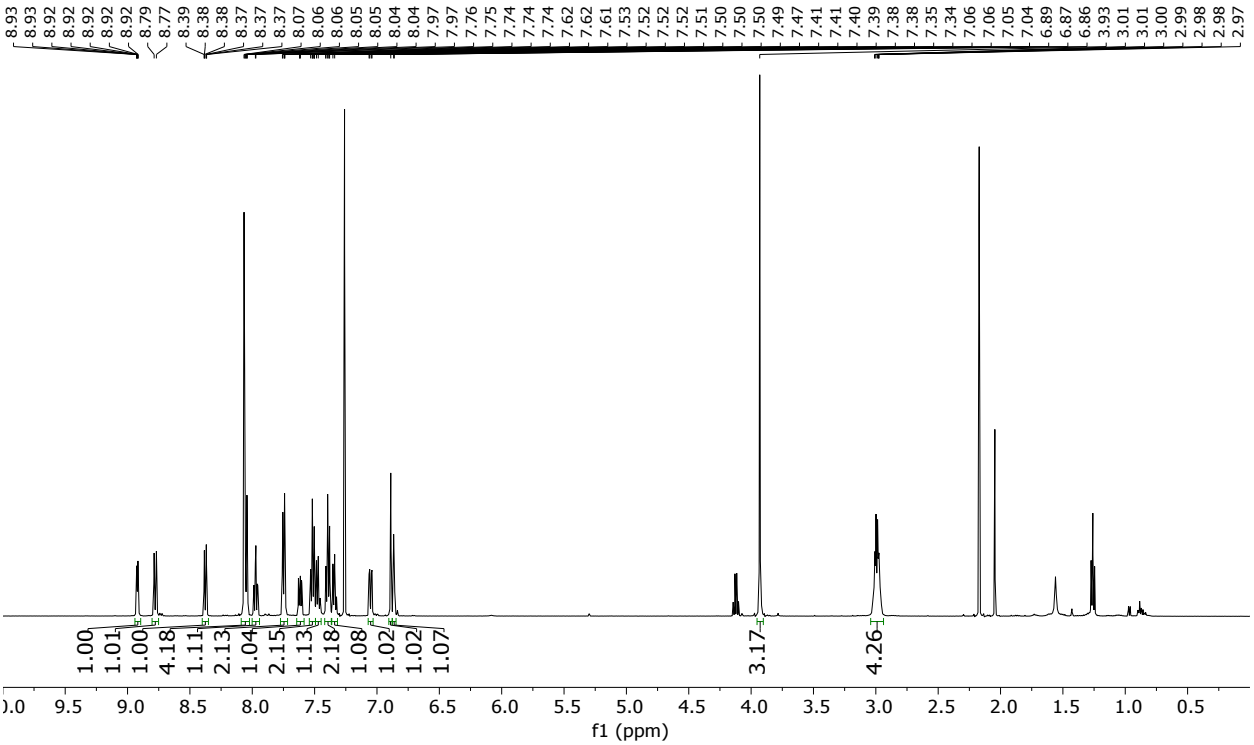


Figure S50. ^{1}H NMR of **CRaB-OMe-APC** ($CDCl_3$).

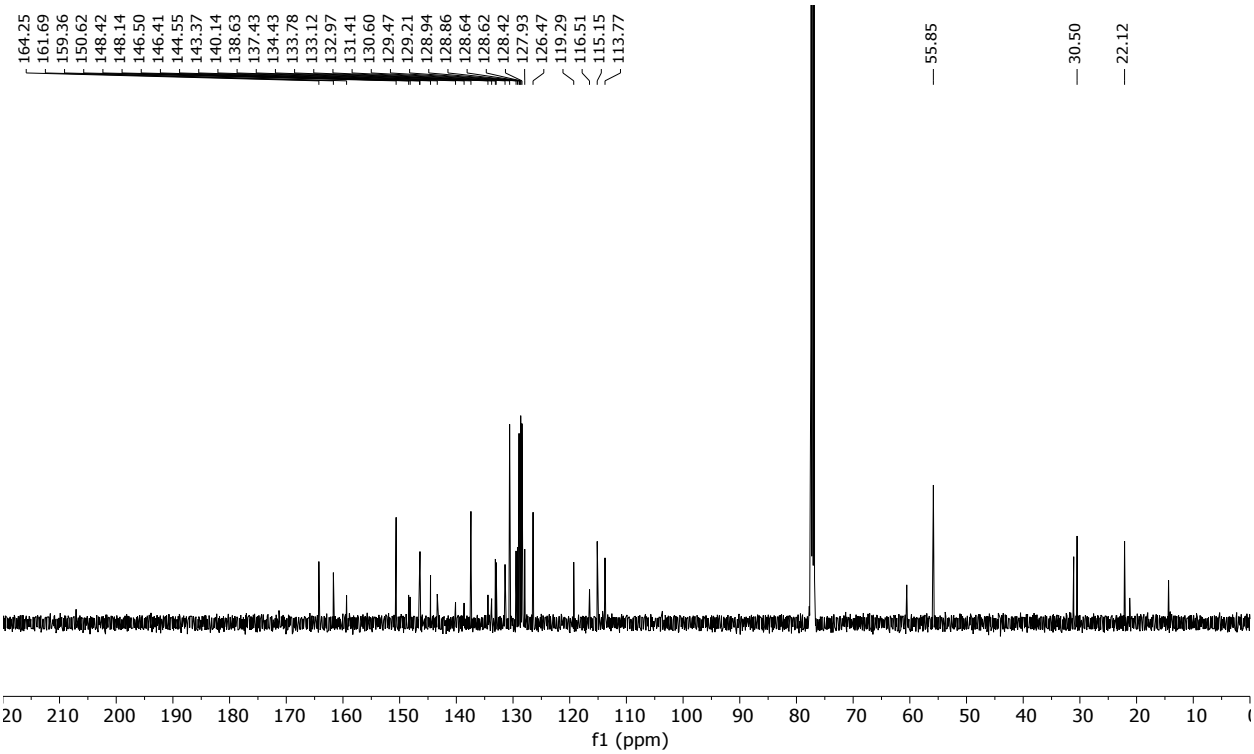


Figure S51. ^{13}C NMR of **CRaB-OMe-APC** ($CDCl_3$).

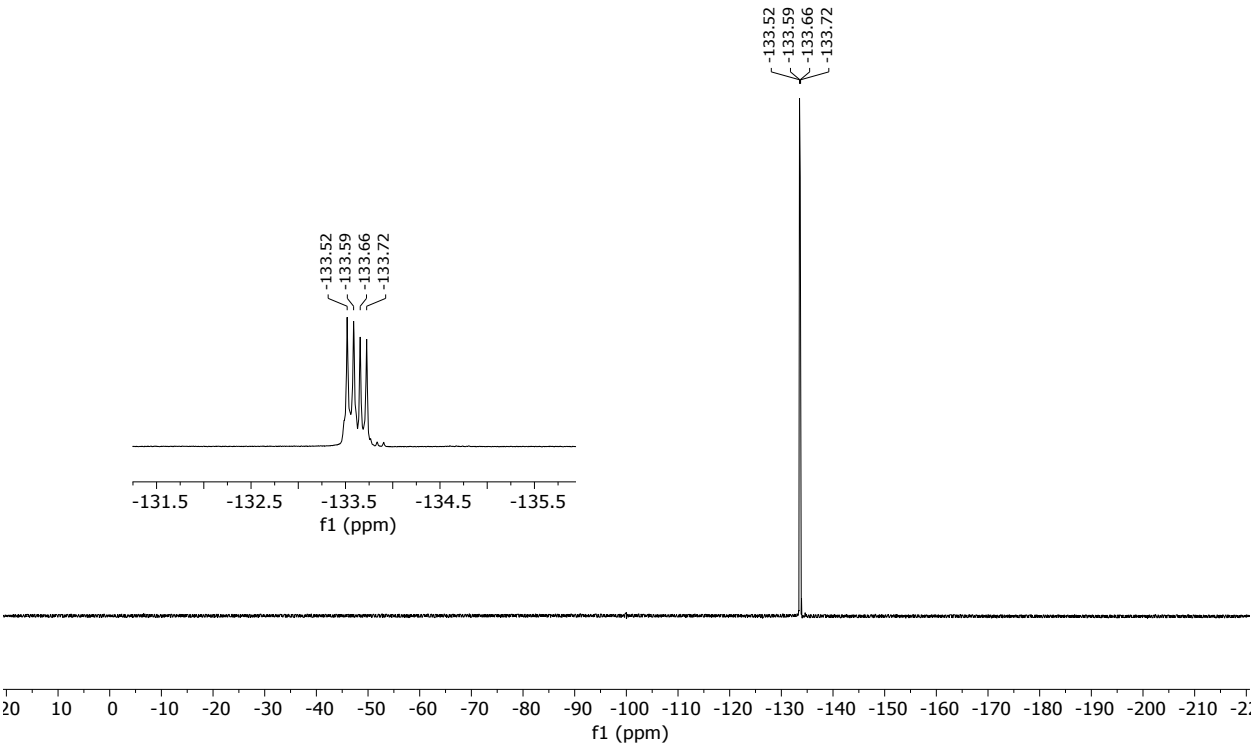


Figure S52. ^{19}F NMR of **CRaB-OMe-APC** ($CDCl_3$).

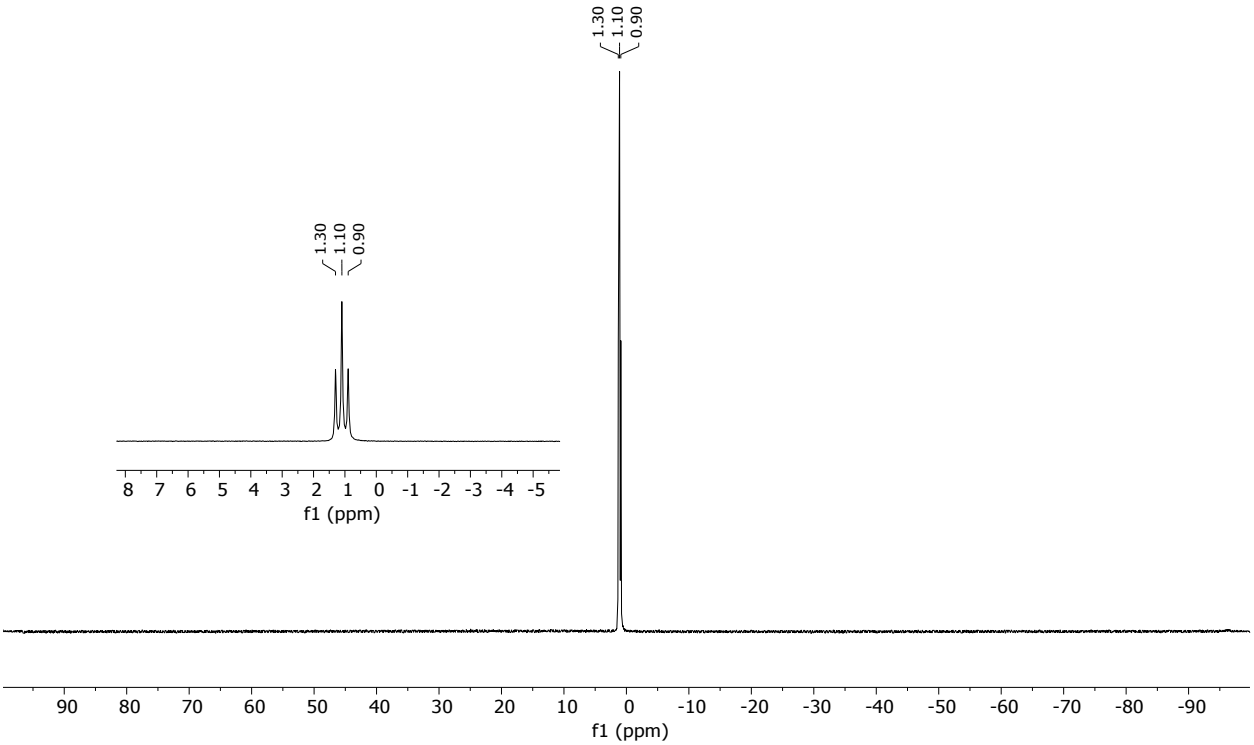


Figure S53. ^{11}B NMR of **CRaB-OMe-APC** ($CDCl_3$).

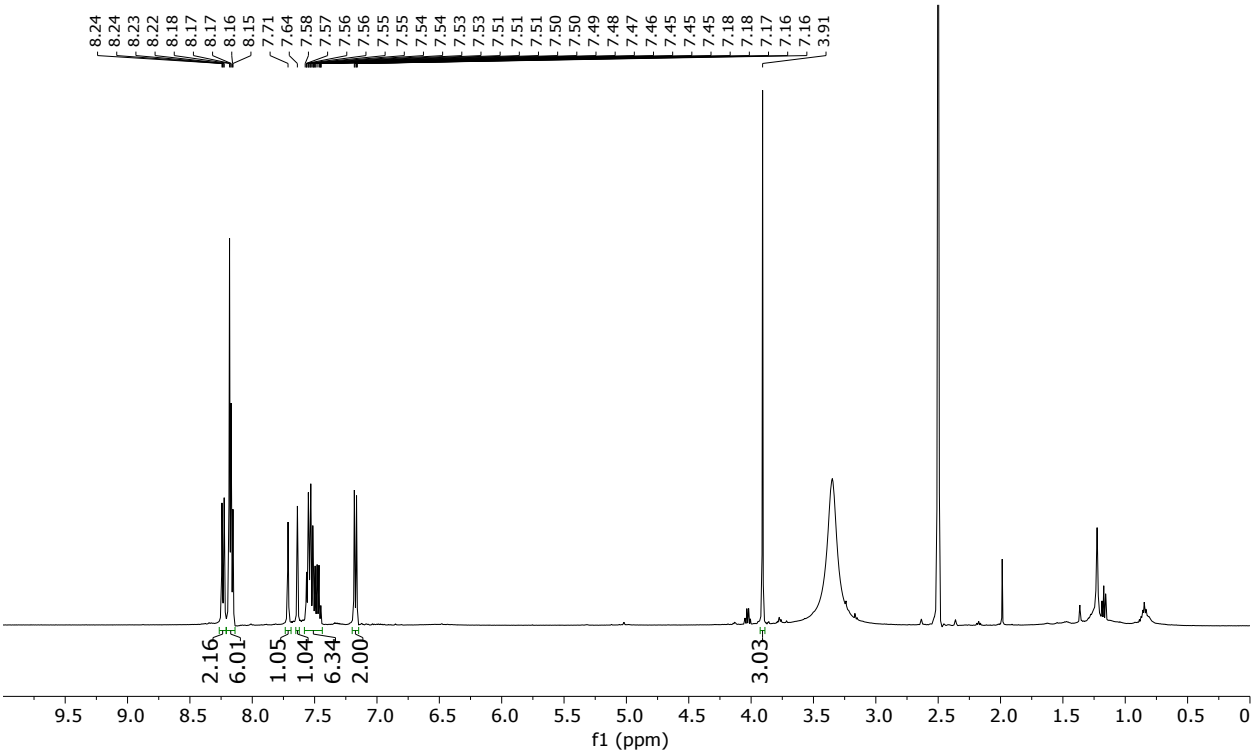


Figure S54. ^{1}H NMR of **t-OMe-APC** (DMSO-d_6).

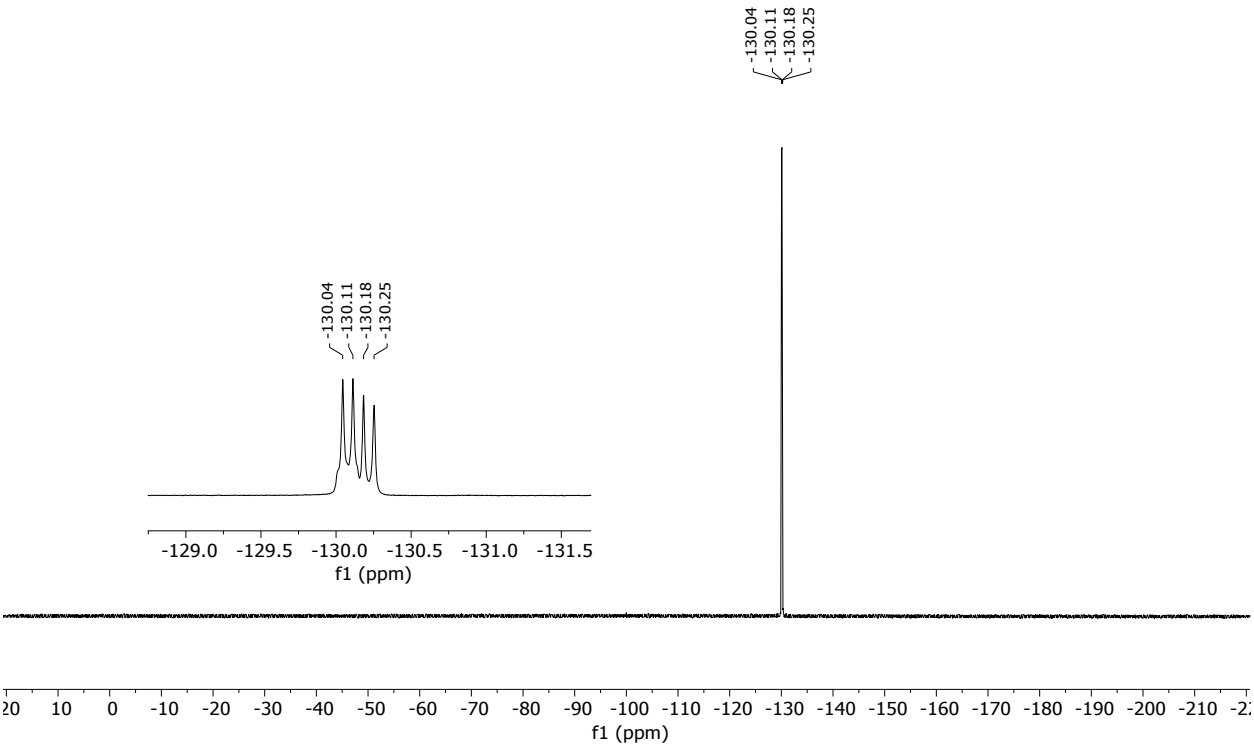


Figure S55. ^{19}F NMR of **t-OMe-APC** (DMSO-d_6).

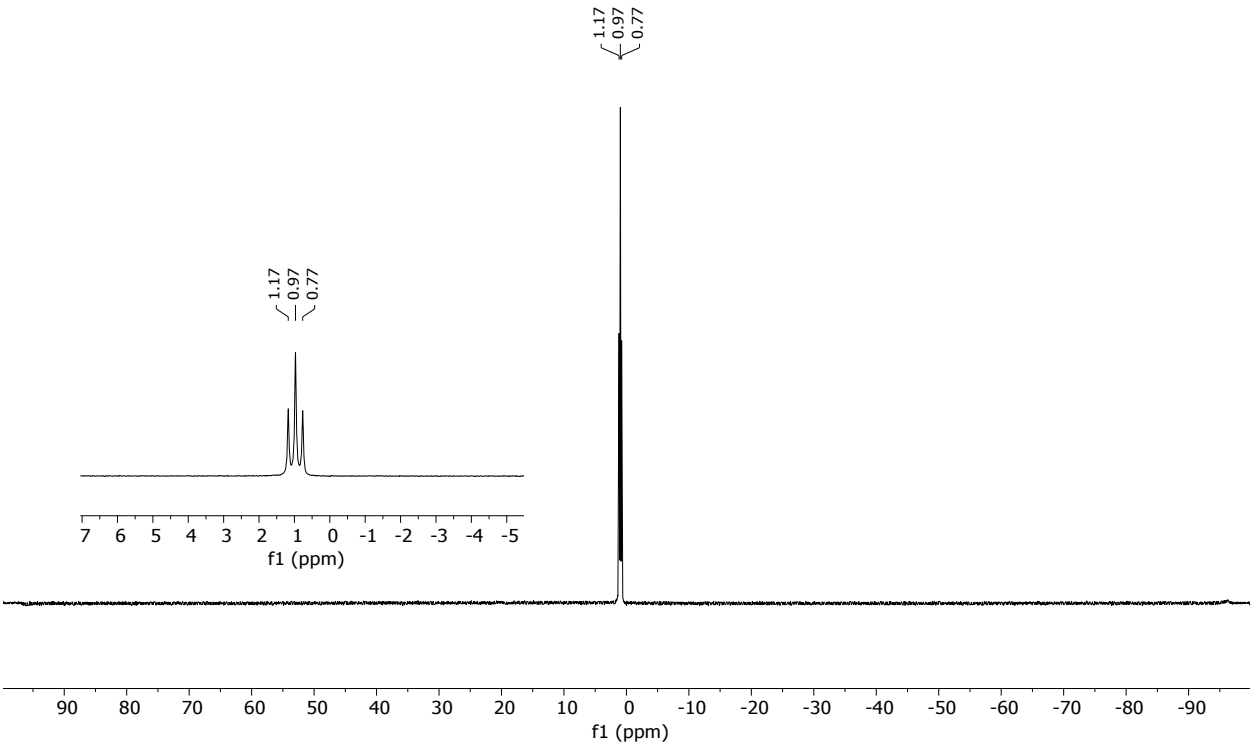


Figure S56. ^{11}B NMR of **t-OMe-APC** (DMSO-d_6).

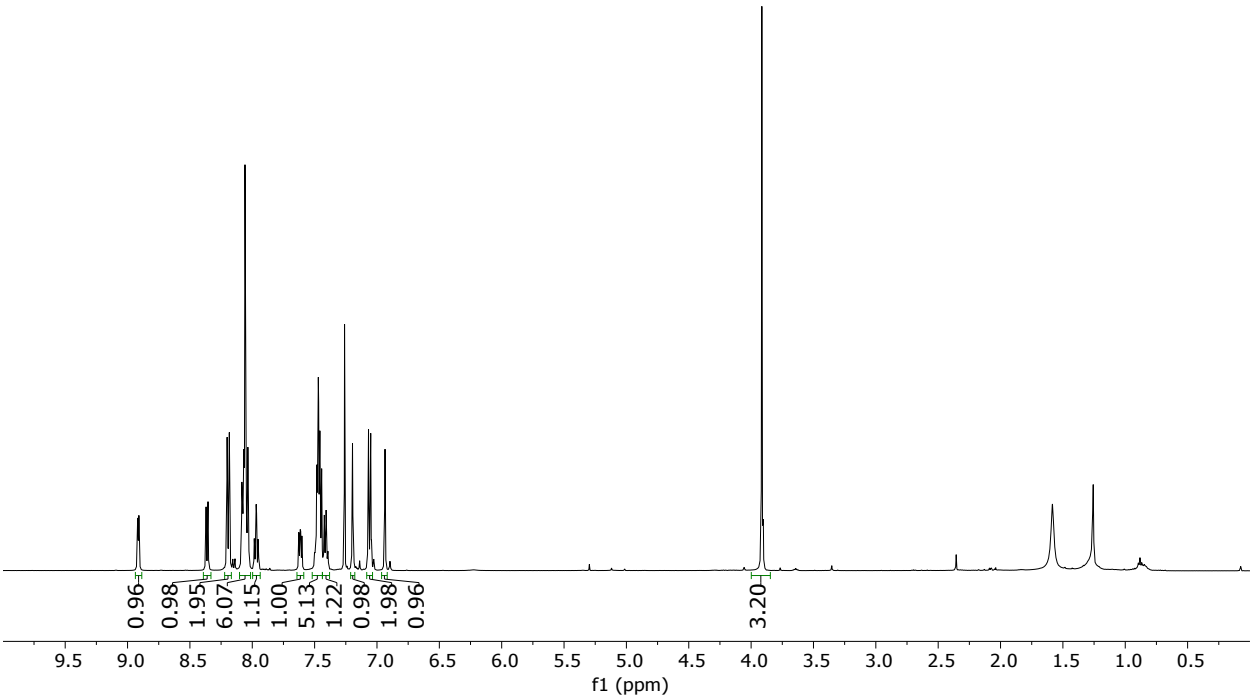


Figure S57. ^{1}H NMR of **OMe-APC** ($CDCl_3$).

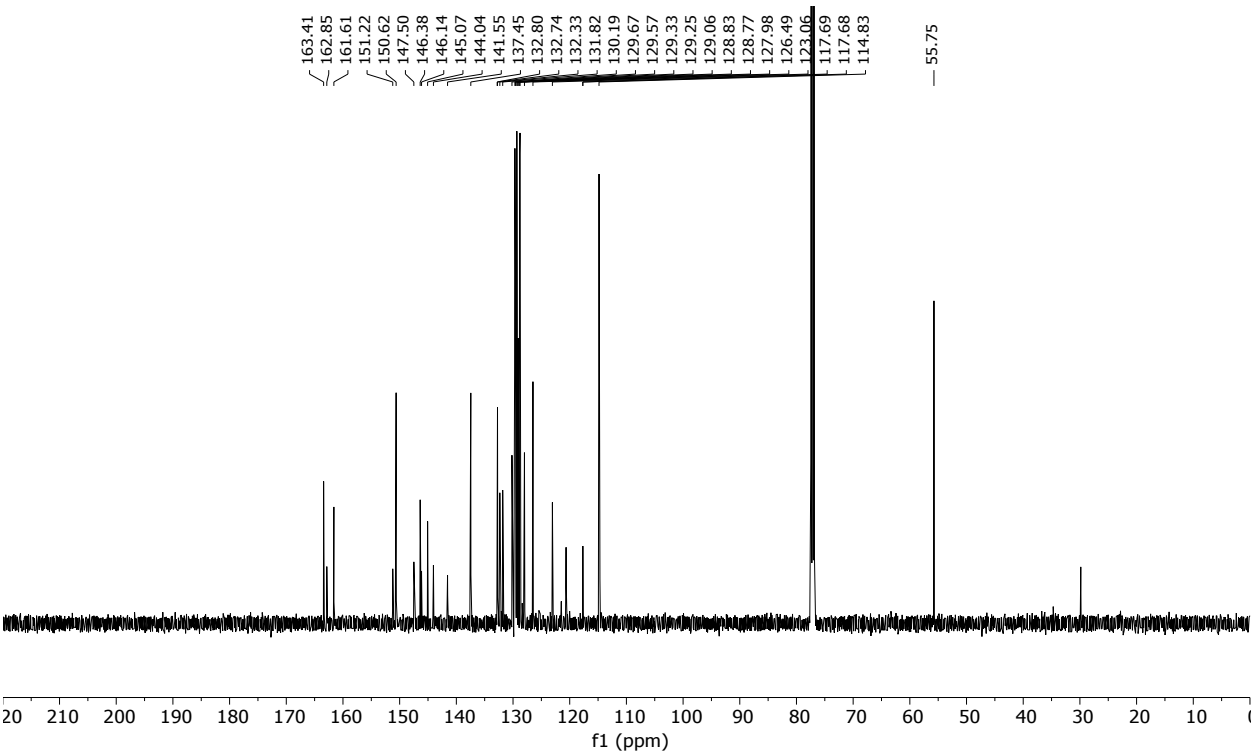


Figure S58. ^{13}C NMR of **OMe-APC** ($CDCl_3$).

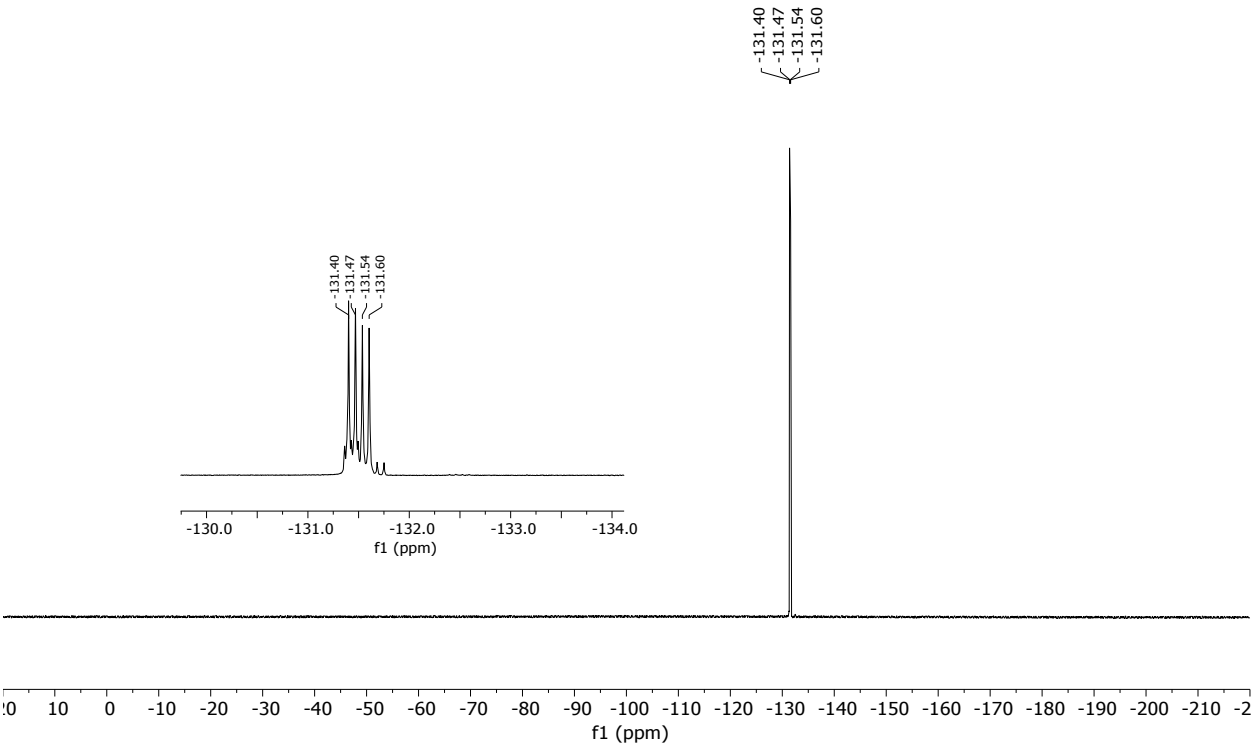


Figure S59. ^{19}F NMR of **OMe-APC** ($CDCl_3$).

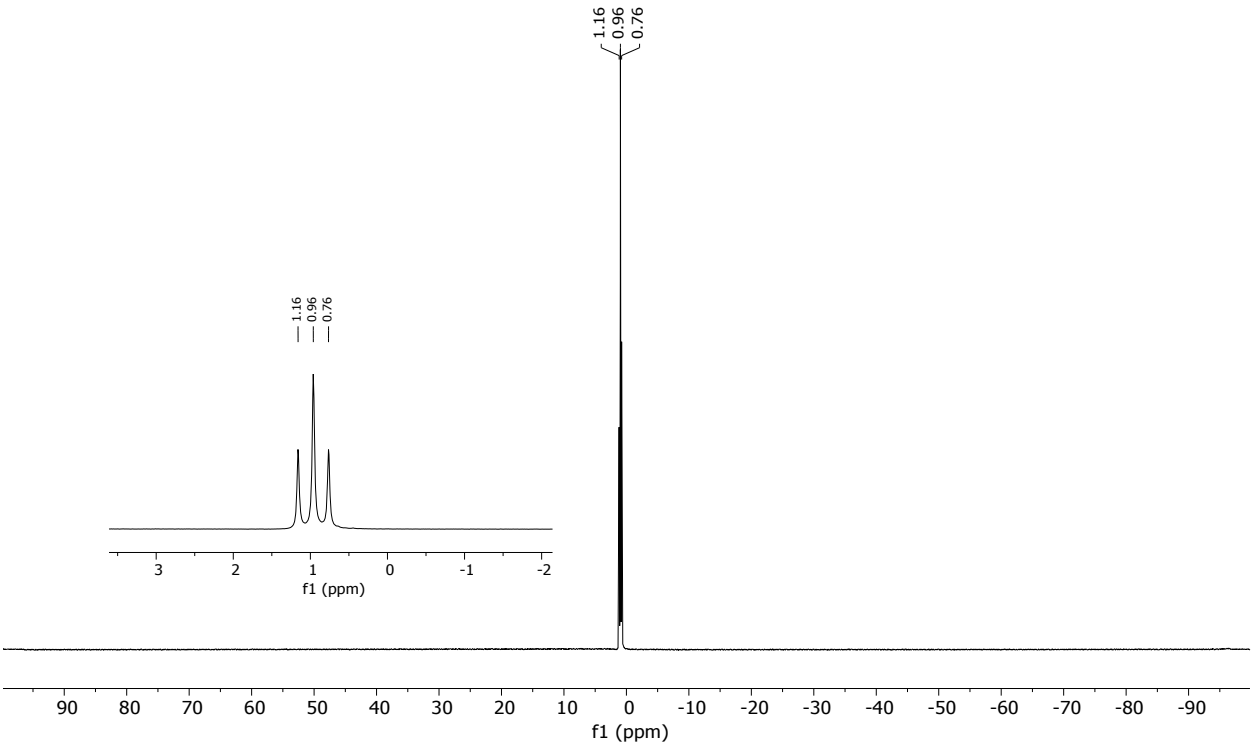


Figure S60. ^{11}B NMR of **OMe-APC** ($CDCl_3$).