Supporting Information

Dark Hydrazone Fluorescence Labeling Agents Enable Imaging of Cellular Aldehydic Load

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Experimental Methods

Instrumentation. ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian Innova 500 MHz and Varian Mercury 400 MHz NMR spectrometer. ^{1}H- and ^{13}C-NMR Spectra were internally referenced to the residual solvent signal. High-resolution mass spectrometry analysis was performed by the Vincent Coates Foundation Mass Spectrometry Laboratory at Stanford University. Semi-preparative high performance liquid chromatography was performed on a LC-20AD Shimadzu liquid chromatography system, equipped with a SPD-M20A diode array detector and a CBM-20A system controller and using reverse phase (C18) columns. Fluorescence measurements were performed on a Fluorolog 3 Jobin Yvon fluorophotospectrometer equipped with an external temperature controller. Cell imaging was performed on a Nikon Eclipse 80i epifluorescence microscope equipped with Nikon Plan Fluor 20, 40 and 100× objectives and a QIClick™ digital CCD camera. Confocal imaging was performed with Leica SP8 White Light Confocal in the Stanford Microscope Facility. Flow cytometry was performed using a Becton Dickinson FACSAria II instrument, equipped with a UV laser, and analyzed on FlowJo (Tree Star, Inc.). Substrate microplate screening was performed with Fluoroskan Ascent™ Microplate Fluorometer.

Chemicals. Chemicals were purchased from Sigma Aldrich and used without purification unless noted otherwise. 3,4,5-trifluoronitrobenzene was purchased from Combi-Blocks. 3,5-dimethoxyaniline was purchased from Chem Impex International. BODIPY FL and Cy3 NHS-ester were purchased from Lumiprobe. 6-Carboxytetramethylrhodamine NHS-ester and 5-Carboxyfluorescein diacetate NHS-ester were purchased from Berry & Associates. 2,4-dihydroxy-6-methylbenzaldehyde[1], tert-butyl 2-(pyridin-2-yl)hydrazine-1-carboxylate NHS-ester[2] (**2a**) and 7-diethylaminocoumarin (DEAC) NHS-ester[3] were synthesized based on published procedures. 2,4-dimethoxyaniline, p-methoxyaniline, dimethyl-4-phenylenediamine were purchased from TCI America. (2-amino-5-methylphenyl)phosphonic acid was synthesized following published procedures.[4] Daidzin was purchased from Tszchem. EMEM cell culture medium was purchased from American Type Culture Collection. HyClone RPMI-1640 cell culture medium and HyClone penicillin-streptomycin 100× solution were obtained from GE Healthcare. One

Shot™ Fetal Bovine Serum (FBS) was purchased from ThermoFisher Scientific. Both culture media were modified by addition of 10% v/v FBS and 1% v/v HyClone penicillin-streptomycin 100× solutions.

Table S1. Excitation and emission wavelengths used to measure kinetics by fluorescence.

Dye	λ_{Abs} (nm)	λ_{Em} (nm)
BODIPY FL	485	515
DEAC	425	480
Cy3	550	570
Fluorescein	492	517
TAMRA	556	579

Catalyst screening. The rate relative to aniline for each catalyst was calculated using initial rates as measured at the 100 sec time point, with the following equation:

$$\frac{Fluorescence\ intensity\ with\ targeted\ catalyst\ at\ 100s - Fluorescence\ intensity\ with\ no\ catalyst\ at\ 0s}{Fluorescence\ intensity\ with\ aniline\ at\ 100s - Fluorescence\ intensity\ with\ no\ catalyst\ at\ 0s}$$

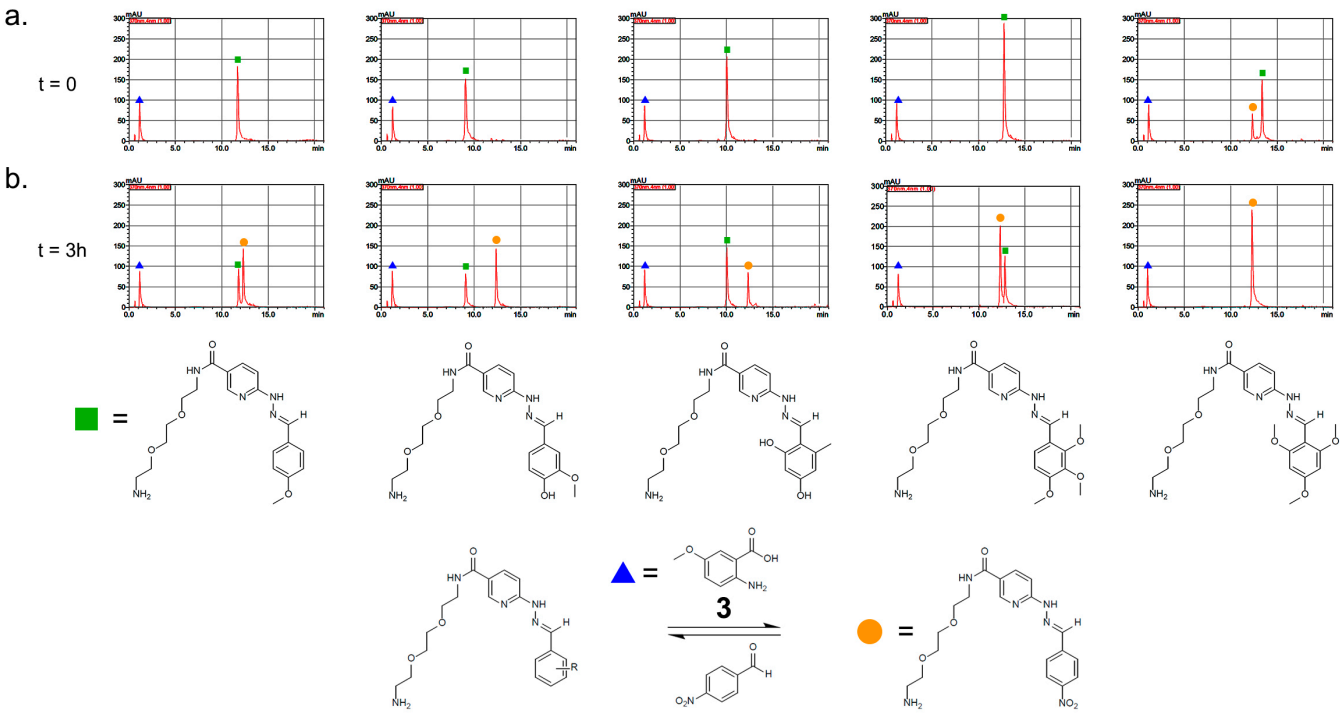


Figure S1. HPLC chromatograms monitoring exchange of various electron-rich hydrazones. Each reaction contains 200 µM starting hydrazones (green squares), 5 mM catalyst **3** (blue triangles) and 500 µM 4-nitrobenzaldehyde. The product hydrazone is indicated by orange circles on the chromatograms. Notice that an observable amount of product hydrazone was formed between reagent mixing and injection into the HPLC for 2,4,6-trimethoxybenzaldehyde hydrazone. The reactions were monitored at bisaryl hydrazone absorption (370 nm).

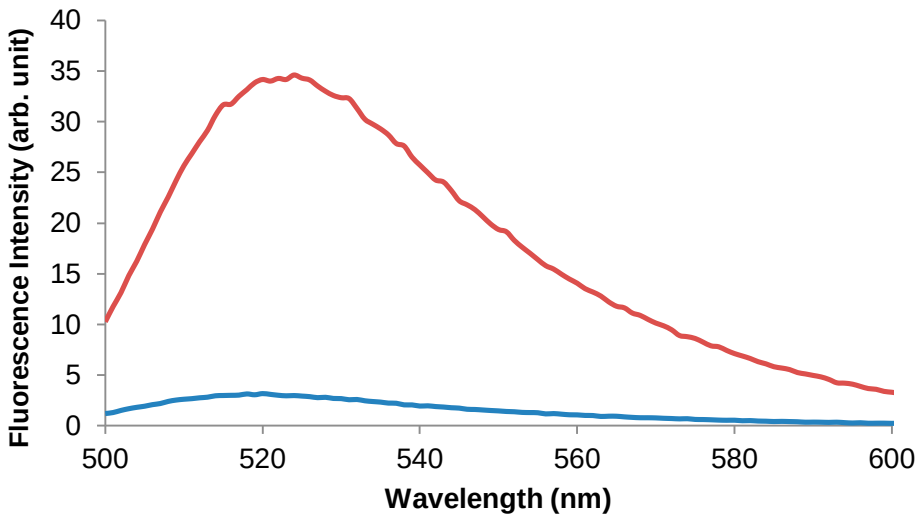


Figure S2. Representative fluorescence emission spectra before (blue) and after (red) hydrazone transfer. The reaction was performed with 500 nM fluorescein DarkZone dye, 5 mM catalyst **3**, and 2 mM glyoxylic acid in 300 mM phosphate buffer until no further fluorescence increase was observed.

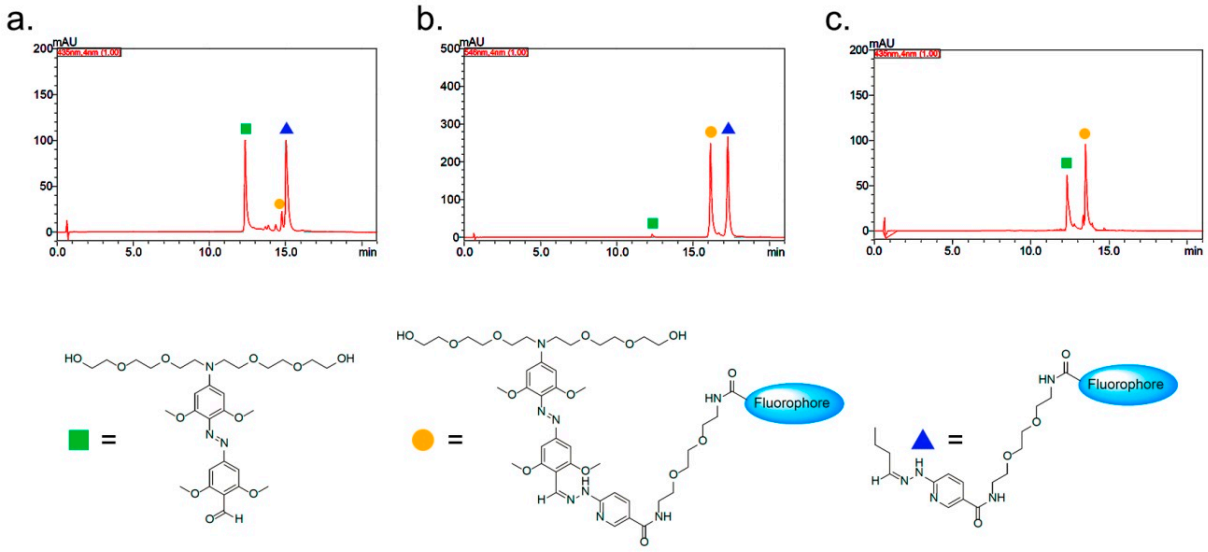


Figure S3. Representative HPLC chromatograms for exchange reactions of a) DEAC DarkZone dye, b) Cy3 DarkZone dye and c) fluorescein DarkZone dye with butyraldehyde. Reactions were performed with 50 µM DarkZone dye, 5 mM catalyst **3**, and 500 µM butyraldehyde in 300 mM phosphate buffer at pH 7. The reaction was injected into HPLC after 21 h of incubation at RT and monitored at the absorption maximum of each fluorophore. Note that fluorescein showed minimal absorption in the HPLC solvent system; the dabcyl absorption maximum was used instead.

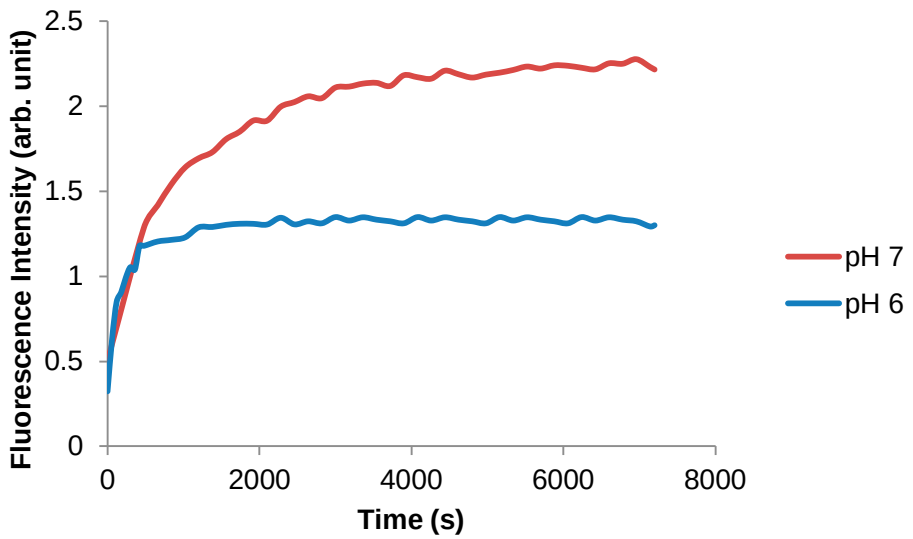


Figure S4. Representative fluorescence exchange kinetic traces of fluorescein DarkZone dye (conditions: 5 µM fluorescein DarkZone dye, 5 mM catalyst **3** and 500 µM aldehyde). Note that fluorescein shows lower emission intensity at pH 6 due to its ring closed form. The fluorescence reaches plateau at ca. 3 h at pH 7 and 20 min at pH 6.

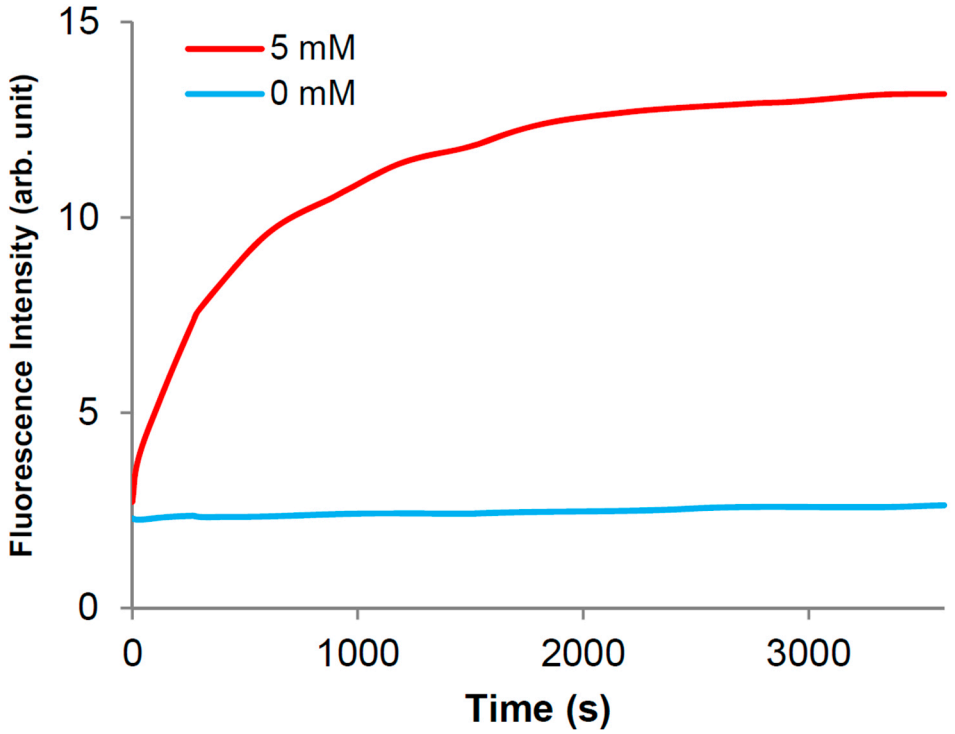


Figure S5. Fluorescence enhancement of catalyzed (5 mM catalyst **3**, red curve) and uncatalyzed (blue curve) DarkZone labeling with 500 nM fluorescein DarkZone dye and 2 mM glyoxylic acid.

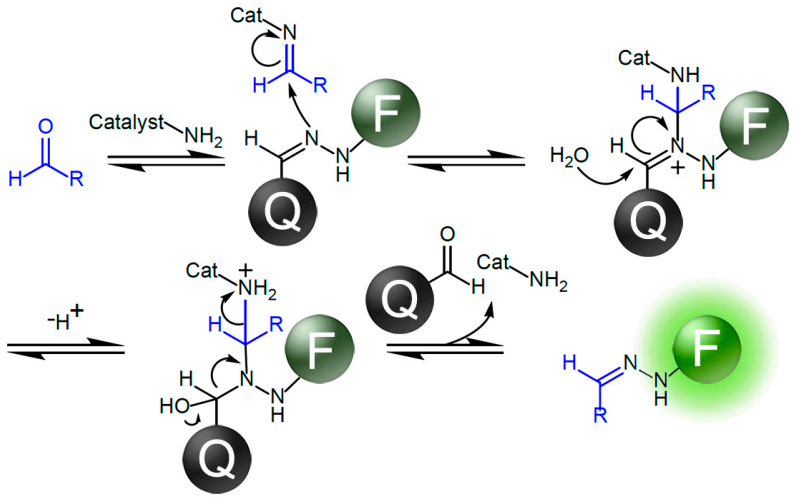


Figure S6. Proposed mechanism of DarkZone labeling.

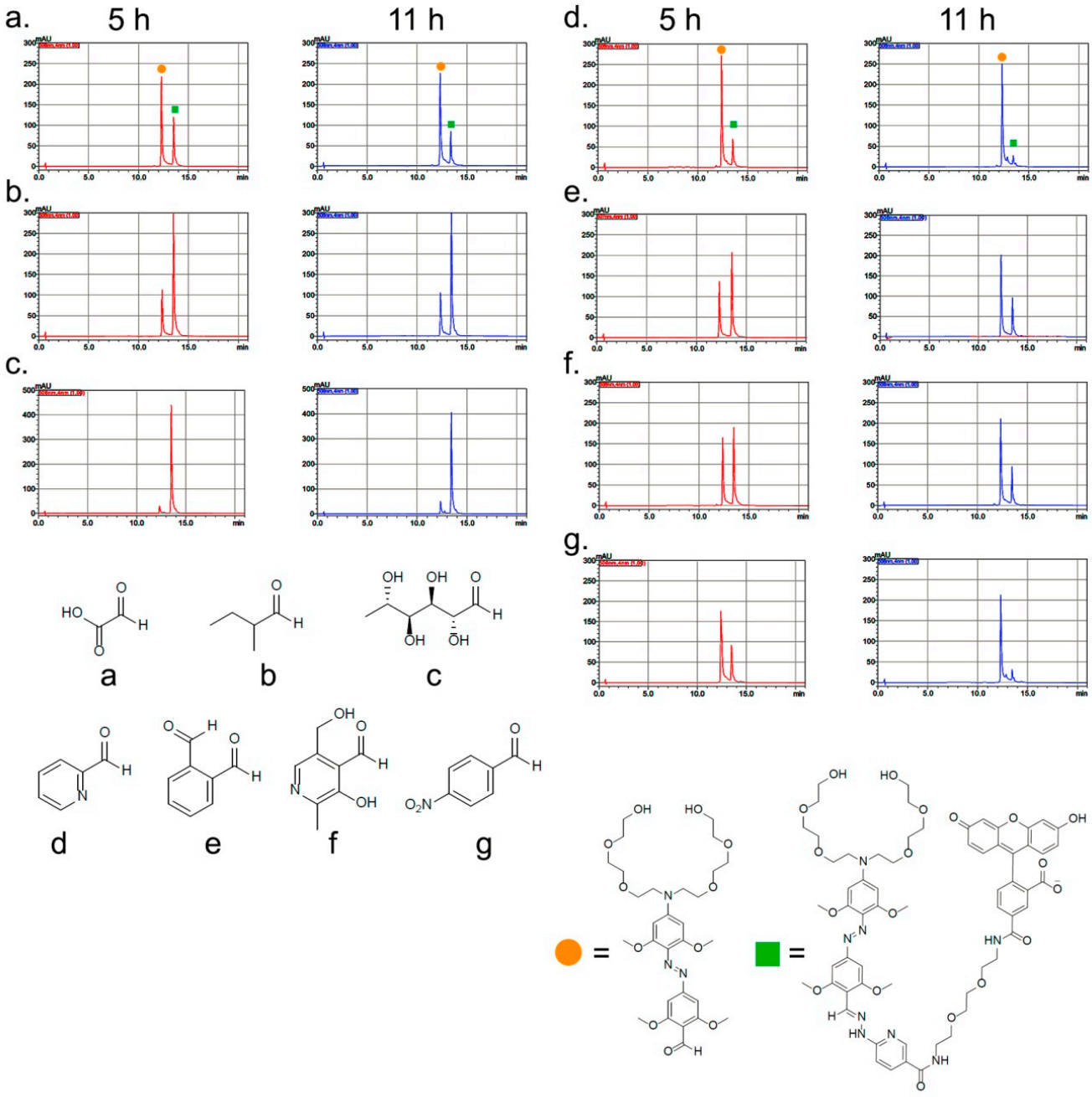


Figure S7. HPLC traces for reaction of various aldehyde substrates. Reactions were performed with 200 µM fluorescein DarkZone dye, 5 mM catalyst **3**, and 200 µM aldehydes and the reactions were injected into HPLC after 5 h and 11 h. The reactions are monitored by HPLC using dabcyl absorption (507 nm). The structures of each aldehyde are labeled with the letters according to the chromatograms. The peaks correspond to the fluorescein DarkZone dye and the released dabcyl were marked as green squares and orange circles, respectively, on the HPLC chromatograms.

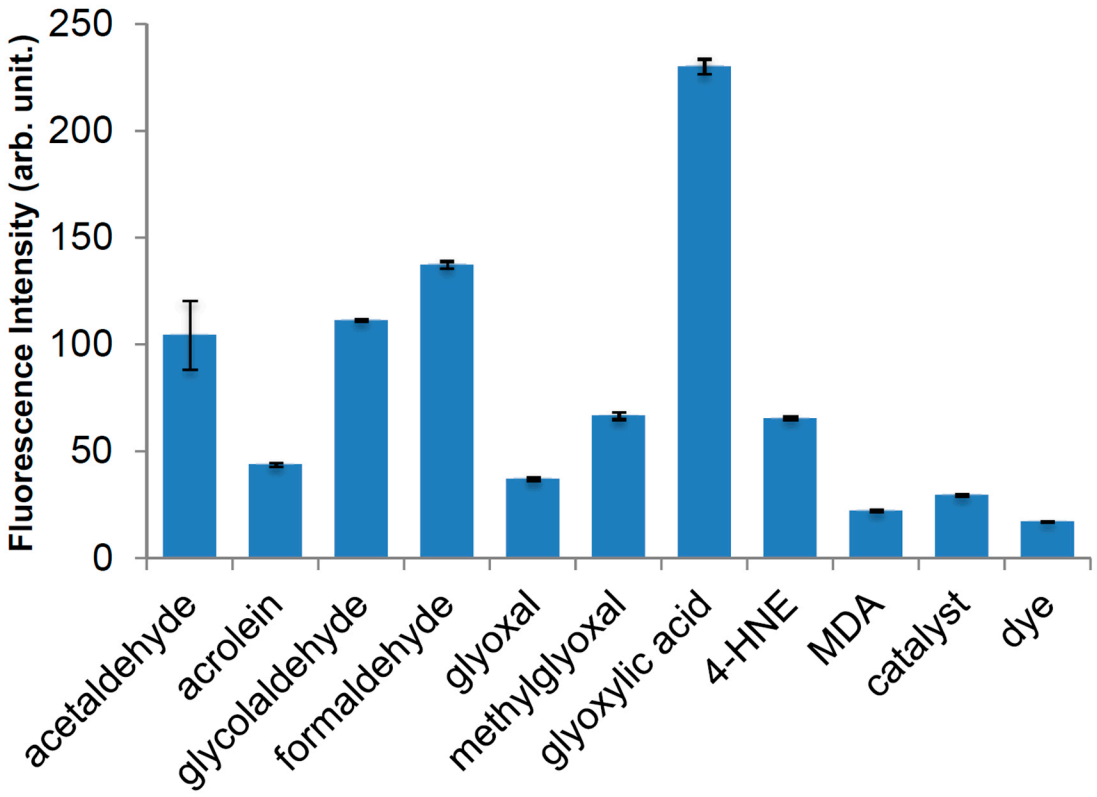
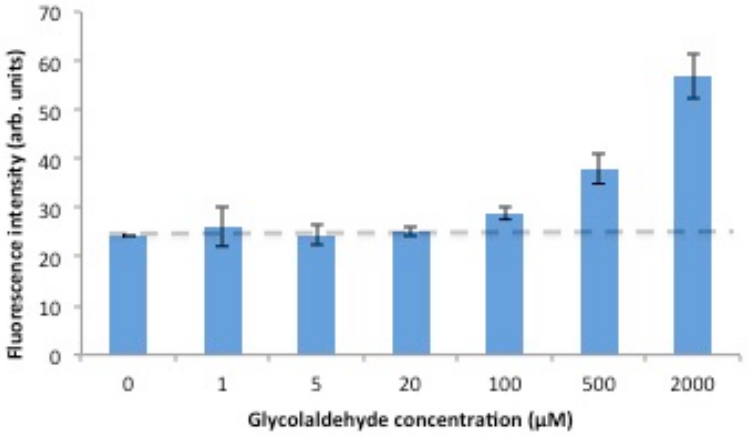


Figure S8. DarkZone labeling of a broad range of biologically relevant alkyl aldehydes. DarkZone labeling was performed with 500 nM fluorescein DarkZone dye, 10 mM catalyst **5** and 2 mM aliphatic aldehyde measured using a microplate reader after 1 h. Only malondialdehyde (MDA) gives no signal over background. Last two lanes are controls; "catalyst" indicates incubation of DarkZone dye with catalyst and "dye" indicates DarkZone dye incubated alone in buffer. Error bars indicate standard deviation over three trials.

a.



b.

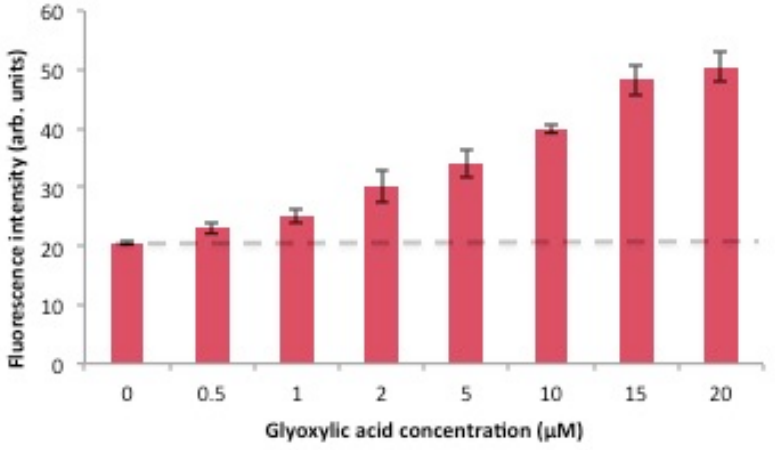


Figure S9. Testing limit of detection for two cellular aldehydes in vitro. (a) glycolaldehyde; (b) glyoxylate. Conditions: 20 µM fluorescein-DarkZone dye, phosphate-buffered saline (pH 7.4), 1 hour incubation.

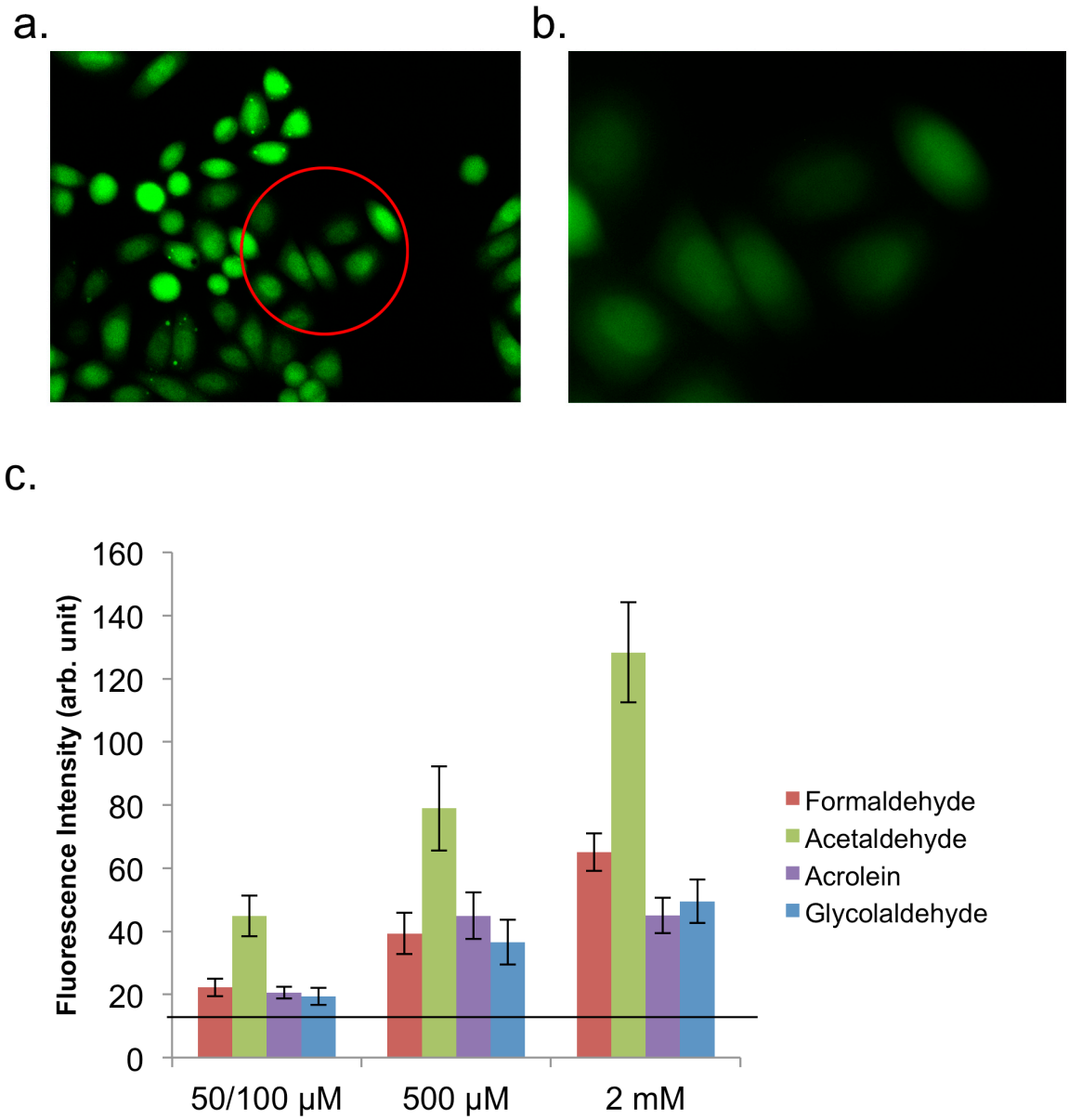


Figure S10. Fluorescence images of HeLa cells treated with 20μM AFDZ dye, 10 mM catalyst **5** and 2 mM acetaldehyde with (a) 20× objective and (b) 100× objective magnification of the region indicated by the red circle. (c) Quantitative fluorescence signals from AFDZ dye in HeLa cells after incubation with increasing concentrations of aldehydes. Data represent averaged fluorescence emission of 10 brightest cells per image (from epifluorescence images in Fig. 5, main text). The line represents the background fluorescence signal with no added aldehyde.

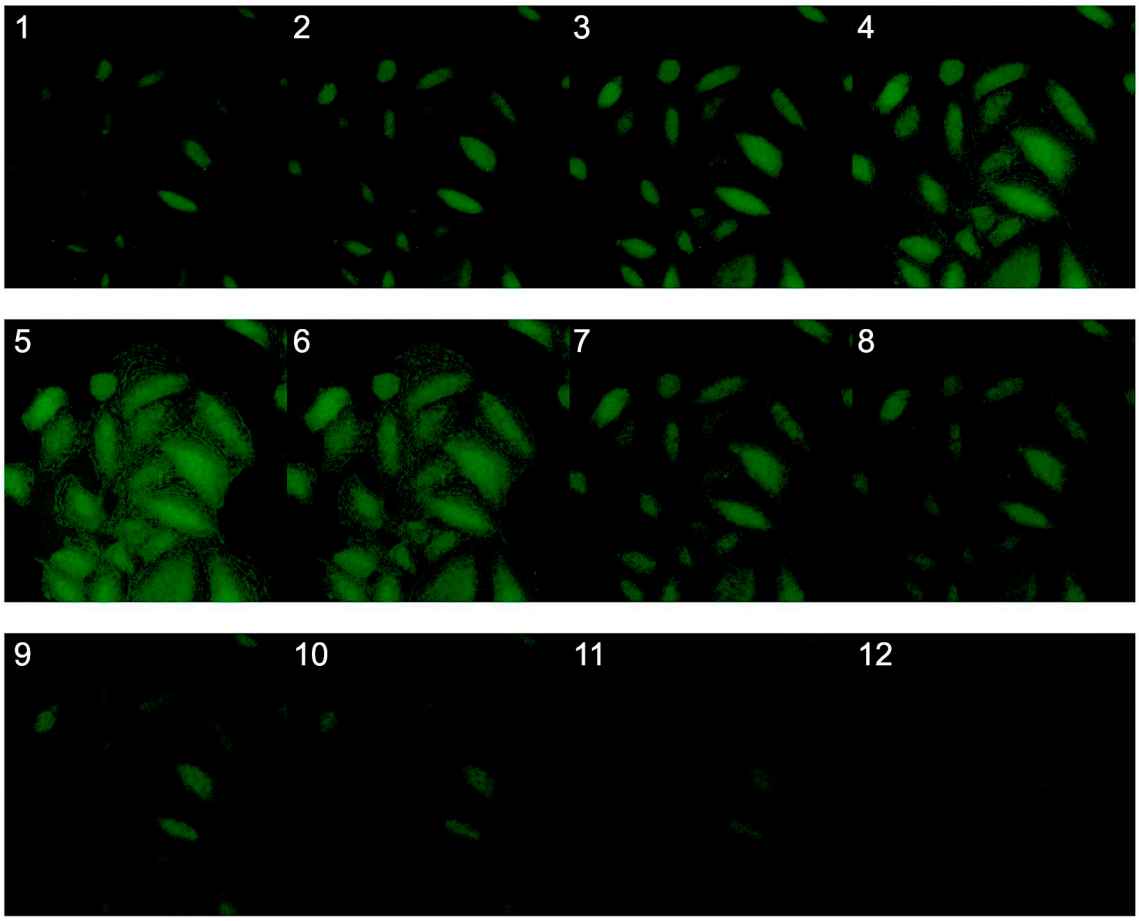


Figure S11. Z-stack of confocal images from HeLa cells incubated with AFDZ dye (20 µM), catalyst **5** and formaldehyde (2 mM) for 1 h, showing localization of dye inside cells. Images were collected at 1.2 µm z-intervals with a 499 nm laser excitation.

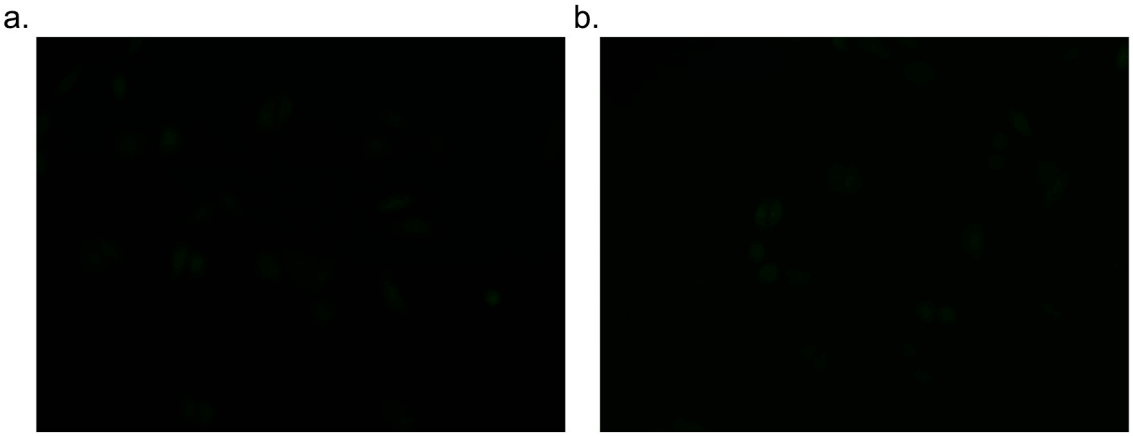


Figure S12. Low background signal in HeLa cells with wild-type ALDH2, where constitutive aldehyde concentrations are expected to be low. AFDZ treatment of HeLa cells with a) 10 mM catalyst **5** and b) 10 mM catalyst **5** and 2 mM glyoxylic acid. The lack of signal in a) demonstrates the hydrolytic stability of AFDZ, while the lack of extracellular signal in b) shows that the AFDZ dye cannot yield signals without intracellular esterase activation.

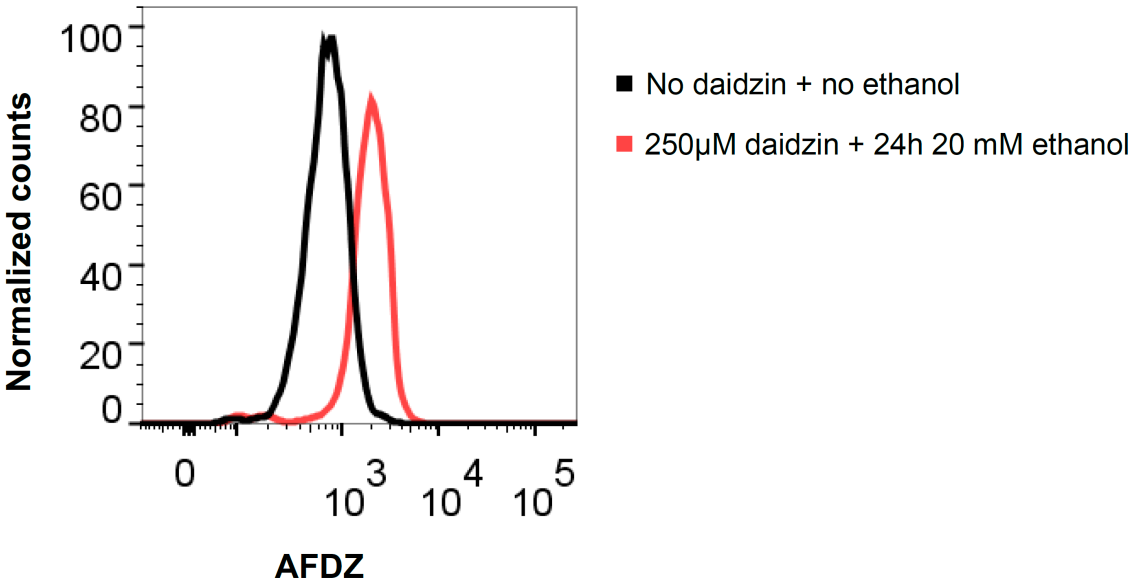
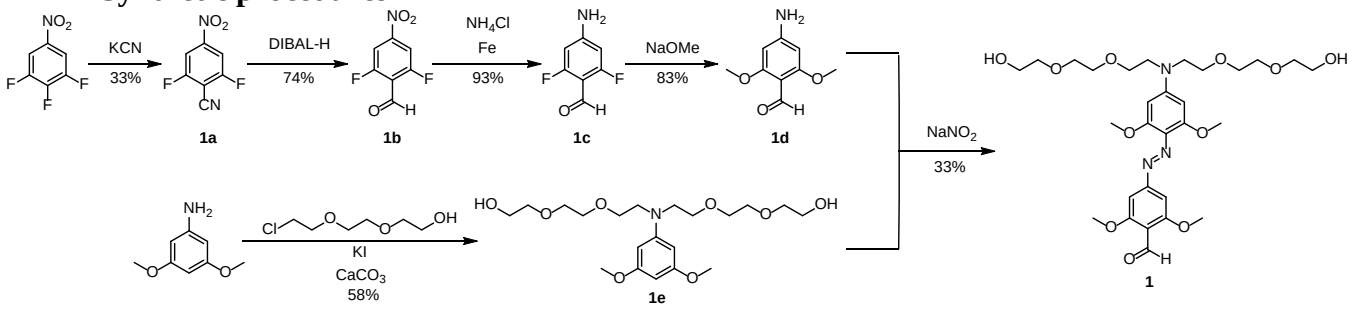
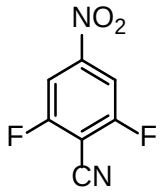


Figure S13. Representative flow cytometry histogram of K562 cells showing acetaldehyde detection from ethanol metabolism using AFDZ dye. Red histogram: cells treated with both daidzin and 20 mM ethanol (24 h) and black histogram: no daidzin and ethanol treatment.

Synthetic procedures





2,6-difluoro-4-nitrobenzonitrile (**1a**)

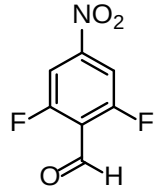
3,4,5-trifluoronitrobenzene (10 g, 56.5 mmol) was dissolved in anhydrous DMSO (90 mL) in a 250 mL round bottom flask and potassium cyanide (3.86 g, 59.3 mmol) was added to the solution ,which was then stirred at room temperature. The exothermic reaction was placed on an ice bath and stirred for 25 min. 90 mL of water was added to the mixture, which was then extracted 4× with diethyl ether (100 mL). The combined organics were washed with 40 mL brine, dried with Na_2SO_4 and evaporated to dryness. The product was purified by flash column chromatography (2:100 hexanes: ethyl acetate to elute the starting material and 5:100 hexanes: ethyl acetate to elute product). The product was obtained as a white solid (3.38 g, 33% yield).

^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.97 (d, *J* = 7.6 Hz, 2H).

^{13}C NMR (126 MHz, Chloroform-*d*) δ 163.36 (dd, *J* = 266.6, 4.5 Hz), 151.74 (t, *J* = 9.9 Hz), 108.67 (dd, *J* = 24.9, 4.4 Hz), 98.81 (t, *J* = 19.4 Hz).

^{19}F NMR (376 MHz, Chloroform-*d*) δ -98.17 (d, *J* = 7.5 Hz).

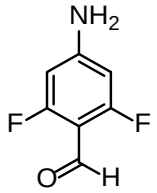
HRMS was attempted but no result was obtained



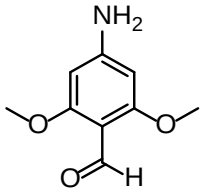
2,6-difluoro-4-nitrobenzaldehyde (**1b**)

1a (1.1 g, 6.02 mmol) was dissolved in anhydrous toluene (30 mL) in a 100 mL round bottom flask and cooled to -78°C. DIBAL-H (6.62 mL, 1M in toluene) was added to the solution dropwise and the dry ice bath was removed. The reaction was stirred at room temperature for 20 h and then quenched by adding 5mL methanol. The solution formed a gel-like mixture, which dissolved upon the addition of 1M HCl (6 mL). 5 mL water was added and the aqueous solution was extracted with dichloromethane (2× 15 mL). The organics were combined and washed with brine (10 mL), dried with Na_2SO_4 and evaporated to dryness. The orange oil was purified by flash column chromatography with 20:3 hexanes: ethyl acetate. 0.83 g colorless crystals were obtained (74% yield).

^{1}H NMR (500 MHz, Chloroform-*d*) δ 10.37 (s, 1H), 7.89 (d, *J* = 8.0 Hz, 2H).
^{13}C NMR (126 MHz, Chloroform-*d*) δ 183.24 (t, *J* = 4.3 Hz), 164.07 (d, *J* = 6.2 Hz), 161.94 (d, *J* = 6.2 Hz), 151.55 (t, *J* = 10.8 Hz), 118.31 (t, *J* = 11.2 Hz), 109.16 – 108.78 (m).
^{19}F NMR (376 MHz, Chloroform-*d*) δ -109.64 (d, *J* = 8.3 Hz).
HRMS was attempted but no result was obtained

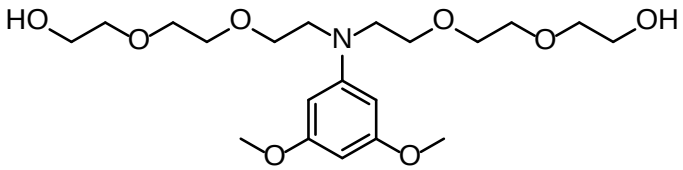


4-amino-2,6-difluorobenzaldehyde (**1c**)
Fe powder (1.2g, <10 µm, 22.2 mmol) and NH_4Cl (4 g, 17.7 mmol) were added to a 100 mL round bottom flask and 12mL of water was added. **1b** was dissolved in 15 mL of methanol and added to the heterogeneous solution above. The solution was stirred vigorously at 80°C for 2 h and then filtered hot and rinsed 3× with boiling methanol. The pale brown filtrate was evaporated and redissolved in ethyl acetate (20 mL). The solution was washed with 10 mL water and the aqueous layer was washed 2× with 15 mL ethyl acetate. The combined organics were washed with 10 mL brine, dried with Na_2SO_4 and evaporated to dryness. The yellow solid was carried on to the next step without further purification (650 mg, 93% yield).
^{1}H NMR (500 MHz, Methanol-d_4) δ 9.84 (s, 1H), 6.13 (d, *J* = 12.5 Hz, 2H)
^{13}C NMR (126 MHz, Methanol-d_4) δ 182.99 (t, *J* = 4.8 Hz), 165.65 (dd, *J* = 257.1, 9.6 Hz), 157.71 (t, *J* = 16.4 Hz), 103.29 (t, *J* = 11.3 Hz), 95.75 (d, *J* = 25.0 Hz).
HRMS [+ Scan]; calculated m/z for $C_7H_5F_2NNaO$ 180.0237; observed mass 180.0234.



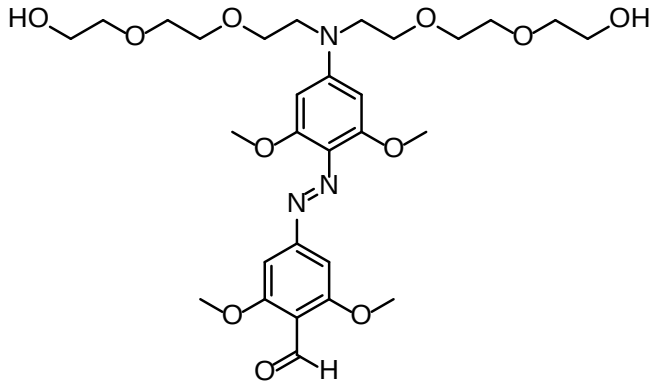
4-amino-2,6-dimethoxybenzaldehyde (**1d**)
1c (650 mg, 4.1 mmol) was dissolved in 5 mL methanol and a sodium methoxide solution was added (4.3 mL, 25 wt%, 20.68 mmol) in 100 mL round bottom flask. The solution was heated to 90°C for 24 h. The volatiles were evaporated until ~2 mL solution remained. 50mL water was added to the mixture and the solid was filtered away.

The filtrate was extracted 3× with ethyl acetate. The organics were combined, dried with Na_2SO_4 and evaporated with silica. The silica was loaded onto a column and purified by flash column chromatography. (1:1 hexanes: ethyl acetate to 100% ethyl acetate. A pale yellow solid was obtained. (0.59 g from filtering and 0.03g from column). The filtered product was sufficiently pure for the next reaction. (620 mg, 83% yield).
^{1}H NMR (500 MHz, DMSO-d_6) δ 10.01 (s, 1H), 6.36 (s, 2H), 5.81 (s, 2H), 3.70 (s, 6H).
^{13}C NMR (126 MHz, DMSO-d_6) δ 184.28 , 164.49 , 157.31 , 104.67 , 89.27 , 55.95
HRMS [+ Scan]; calculated m/z for $C_9H_{11}NNaO_3$ 204.0637; observed mass 204.0637.



9-(3,5-dimethoxyphenyl)-3,6,12,15-tetraoxa-9-azaheptadecane-1,17-diol (**1e**)
3,5-dimethoxyaniline (1g, 6.5 mmol), 2-[2-(2-Chloroethoxy)ethoxy]ethanol (3.29 g, 19.5 mmol), calcium carbonate (2.5g, 25.0 mmol) and potassium iodide (0.11 g, 0.65 mmol) were dissolved in 20 mL 4:1 water: ethanol and the reaction was refluxed at 130°C overnight. 20 mL of water was added and the solution was extracted with ethyl acetate (30 mL × 3). The combined organics were washed with brine (20 mL × 2), dried with $MgSO_4$, evaporated and purified by flash column chromatography. (Ethyl acetate to ethyl acetate with 5% methanol). A yellow oil was obtained (1.59 g, 58% yield).

^{1}H NMR (500 MHz, Chloroform-*d*) δ 5.96 – 5.84 (m, 3H), 3.76 (s, 6H), 3.75 – 3.72 (m, 4H), 3.69 – 3.56 (m, 22H).
^{13}C NMR (126 MHz, Chloroform-*d*) δ 161.58 , 149.16 , 91.33 , 88.03 , 72.44 , 70.33 , 70.19 , 68.44 , 61.46 , 55.04 , 50.99 .
HRMS [+ Scan]; calculated m/z for $C_{20}H_{36}NO_8$ 418.2435; observed mass 418.2437.

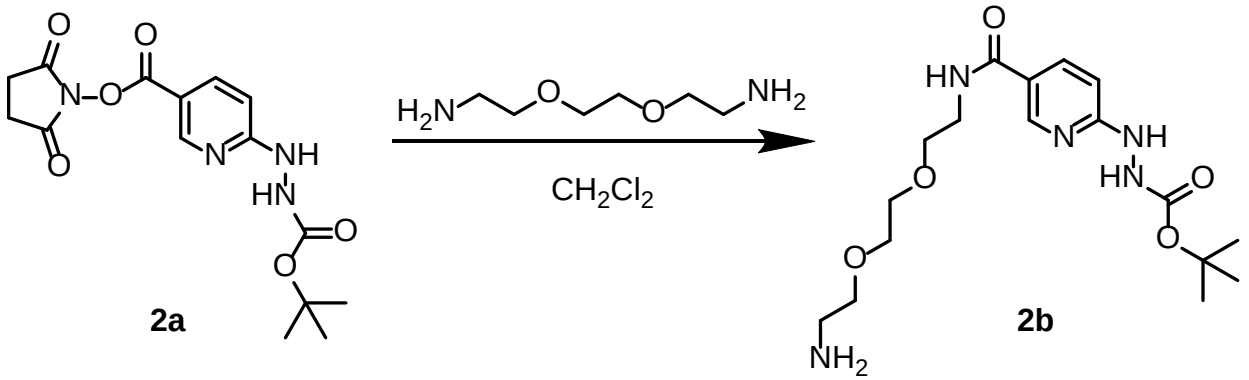


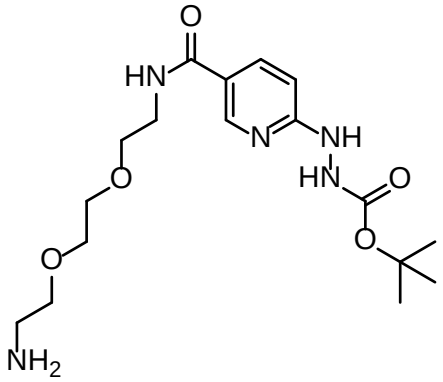
(E)-4-((4-(bis(2-(2-(2-hydroxyethoxy)ethoxy)ethyl)amino)-2,6-dimethoxyphenyl)diazenyl)-2,6-dimethoxybenzaldehyde (**1**)
1d (60 mg, 0.33 mmol) was added to a 25 mL round bottom flask and 3.5 mL of DMF and 0.5 mL 12M HCl were added. The heterogeneous mixture was cooled to 0°C.

Sodium nitrite (28.6 mg 0.41 mmol) was dissolved in 1 mL water and added dropwise to **1d**. The solution was stirred at 0°C for 90 min. The solution became lighter yellow in color over time. **1e** was dissolved with 2 g of sodium acetate in 5 mL of water and it was poured into the diazonium solution. The reaction was warmed to RT and stirred for 2 h.

5 mL of saturated Na_2CO_3 and 10 mL brine were added to the solution and it was then extracted 2× with chloroform (30 mL). The combined organics were dried with Na_2SO_4 and evaporated to dryness. The red solid was purified by flash column (1:1 acetone: CH_2Cl_2 to elute the unreacted starting material and 50:50:3:1 acetone: CH_2Cl_2: methanol: triethylamine) to elute the red product (55.3 mg, 33%).

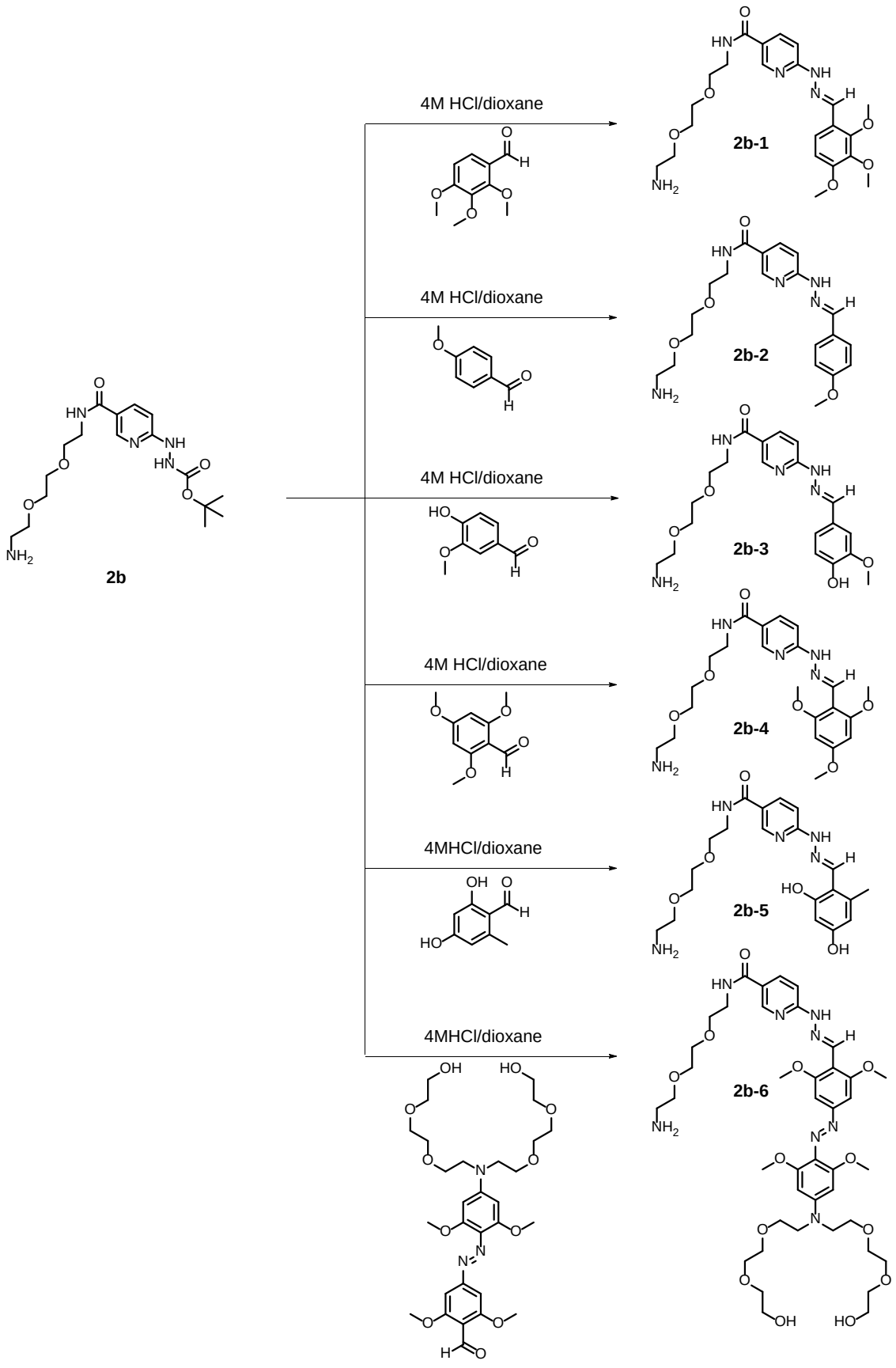
^{1}H NMR (500 MHz, Chloroform-*d*) δ 10.47 (s, 1H), 7.03 (s, 2H), 6.00 (s, 2H), 3.96 (s, 6H), 3.94 (s, 6H), 3.78 – 3.56 (m, 26H).
^{13}C NMR (126 MHz, Chloroform-*d*) δ 189.31 , 163.14 (overlapping peaks) , 158.04 (overlapping peaks) , 124.34 , 113.49 , 97.56 , 89.41 , 72.90 , 70.95 , 70.72 , 69.02 , 61.91 , 56.75 , 56.45 , 51.72 .
HRMS [+ Scan]; calculated m/z for $C_{29}H_{44}N_3O_{11}$ 610.2970; observed mass 610.2968.



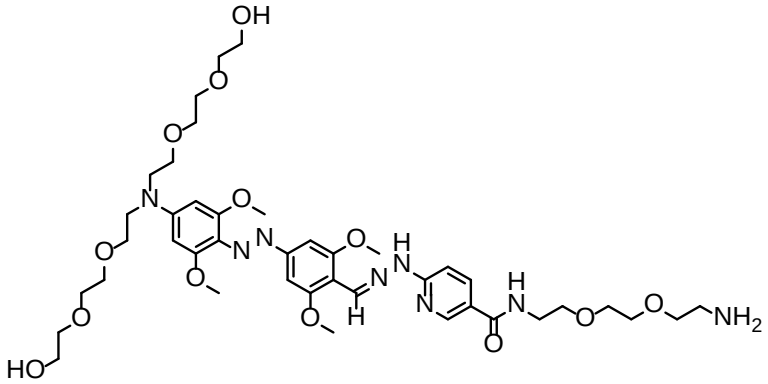


(5-((2-(2-(2-aminoethoxy)ethoxy)ethyl)carbamoyl) 6-Boc-hydrazinonicotinate (**2b**)
2a (578 mg, 0.28 mmol) was dissolved in CH_2Cl_2 (50 mL) and it was added dropwise over 4 h to a solution of 2-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)ethanamine (2.4 g, 16 mmol) dissolved in CH_2Cl_2 (10 mL). The reaction was stirred at RT for 6 h. Solvent was evaporated and the residue redissolved in ethyl acetate (50 mL). The organic was washed with brine (20 mL × 3). The organic layer was collected, dried with $MgSO_4$, filtered and evaporated to dryness. The oil was dissolved in water with white insoluble solid formed, which was removed with a 0.22 µm filter. The aqueous layer was then washed CH_2Cl_2 (20 mL×3). The aqueous later was collected and evaporated to dryness and was used without further purification.

^{1}H NMR (400 MHz, Acetonitrile-d_3) δ 8.49 (s, 1H), 7.90 (d, *J* = 8.9 Hz, 1H), 7.62 (s, 1H), 6.58 (d, *J* = 9.0 Hz, 1H), 3.61 – 3.30 (m, 12H), 2.72 – 2.62 (m, 2H), 1.42 (s, 9H).
^{13}C NMR (100 MHz, Acetonitrile-d_3) δ 166.64 , 162.76 , 157.18 , 148.69 , 137.76 , 122.48 , 105.86 , 81.07 , 73.80 , 70.85 , 70.68 , 70.19 , 42.24 , 40.23 , 28.42 .
HRMS [+ Scan]; calculated m/z for $C_{17}H_{30}N_5O_5$ 384.2241; observed mass 384.2227.


4M HCl/dioxane
4M HCl/dioxane
4M HCl/dioxane
4M HCl/dioxane
4MHCl/dioxane
4MHCl/dioxane
2b
2b-1
2b-2
2b-3
2b-4
2b-5
2b-6

General procedures for hydrazone formation

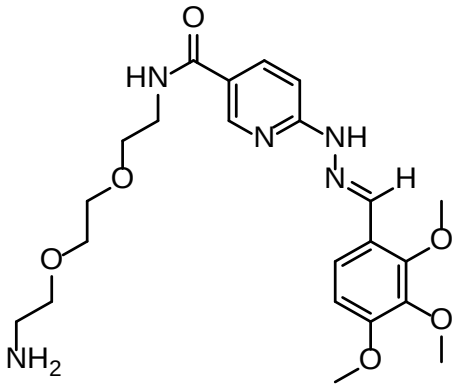


N-(2-(2-(2-aminoethoxy)ethoxy)ethyl)-6-(2-(4-((E)-(4-(bis(2-(2-(2-hydroxyethoxy)ethoxy)ethyl)amino)-2,6-dimethoxyphenyl)diazenyl)-2,6-dimethoxybenzylidene)hydrazinyl)nicotinamide (**2b-6**)

Neat **2b** (10 mg, 26 µmol) was cooled to 0°C and 0.5 mL 4M HCl in dioxane was added. The reaction was stirred at 0°C for 2 h. The volatiles were evaporated. **1** (15.9 mg, 26 µmol) was dissolved in 0.5 mL ethanol and was added to the reaction, followed by 1 µL of 12M HCl. The reaction was heated to 50°C for 4 h. The reaction was evaporated and redissolved in water, filtered using a 0.22 µM filter, and purified by RP-HPLC using a semipreparative C18 column. (16.2 mg, 71% yield)

^{1}H NMR (500 MHz, Methanol-d_4) δ 8.60 – 8.52 (m, 2H), 8.36 (d, J = 9.3 Hz, 1H), 7.25 (d, J = 9.7 Hz, 1H), 7.13 (s, 2H), 6.47 (s, 2H), 4.31 – 4.06 (m, 10H), 4.02 (s, 6H), 3.93 – 3.85 (m, 4H), 3.75 – 3.59 (m, 22H), 3.55 (t, J = 4.9 Hz, 4H), 3.14 (t, J = 5.2 Hz, 2H).

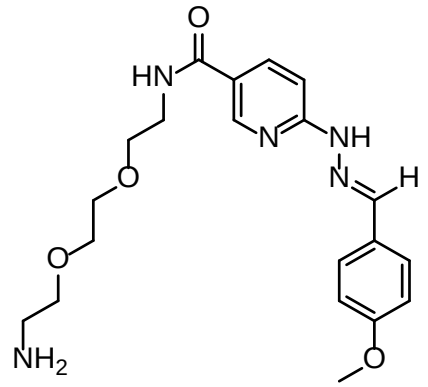
HRMS [+ Scan]; calculated m/z for $C_{41}H_{63}N_8O_{13}$ 875.4509; observed mass 875.4515.



(E)-N-(2-(2-(2-aminoethoxy)ethoxy)ethyl)-6-(2-(2,3,4-trimethoxybenzylidene)hydrazinyl)nicotinamide (**2b-1**)

^{1}H NMR (500 MHz, Methanol-d_4) δ 8.55 (dd, J = 2.1, 0.7 Hz, 1H), 8.51 (s, 1H), 8.38 (dd, J = 9.3, 2.1 Hz, 1H), 7.97 (d, J = 8.9 Hz, 1H), 7.22 (d, J = 9.3 Hz, 1H), 6.93 (d, J = 8.9 Hz, 1H), 3.98 (s, 3H), 3.94 (s, 3H), 3.87 (s, 3H), 3.72 (m, 8H), 3.62 (t, J = 5.7 Hz, 2H), 3.14 (t, J = 5.0 Hz, 2H). (80% yield)

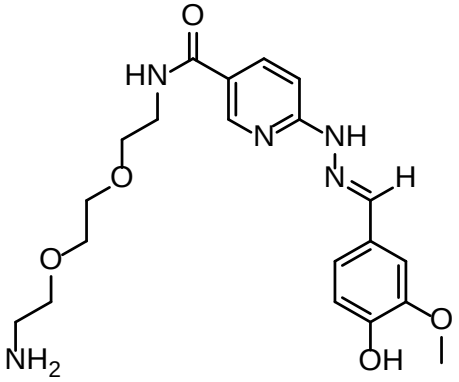
HRMS [+ Scan]; calculated m/z for $C_{22}H_{32}N_5O_6$ 462.2347; observed mass 462.2348.



(E)-N-(2-(2-(2-aminoethoxy)ethoxy)ethyl)-6-(2-(4-methoxybenzylidene)hydrazinyl)nicotinamide (**2b-2**)

^{1}H NMR (500 MHz, Methanol-d_4) δ 8.54 (dd, *J* = 2.1, 0.7 Hz, 1H), 8.41 (dd, *J* = 9.4, 2.1 Hz, 1H), 8.25 (s, 1H), 7.93 – 7.83 (m, 2H), 7.23 (d, *J* = 9.5 Hz, 1H), 7.11 – 6.99 (m, 2H), 3.89 (s, 3H), 3.79 – 3.67 (m, 8H), 3.62 (t, *J* = 5.5 Hz, 2H), 3.14 (t, *J* = 5.0 Hz, 2H). (96% yield)

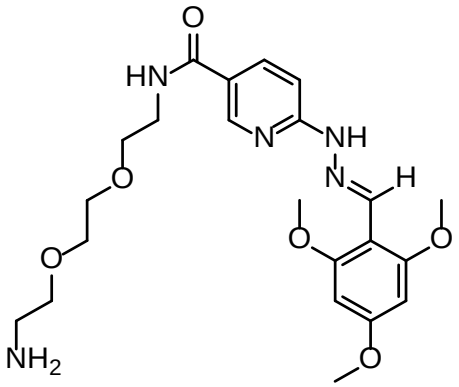
HRMS [+ Scan]; calculated m/z for $C_{20}H_{28}N_5O_4$ 402.2136; observed mass 402.2131.



(E)-N-(2-(2-(2-aminoethoxy)ethoxy)ethyl)-6-(2-(4-hydroxy-3-methoxybenzylidene)hydrazinyl)nicotinamide (**2b-3**)

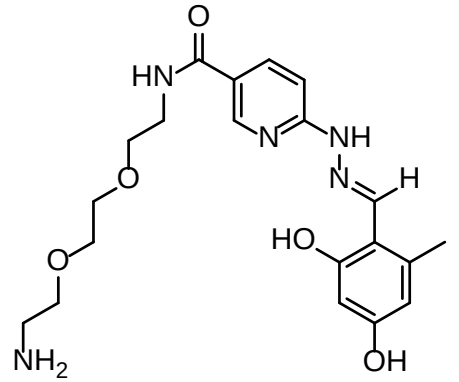
^{1}H NMR (500 MHz, Methanol-d_4) δ 8.52 (dd, *J* = 2.2, 0.7 Hz, 1H), 8.40 (dd, *J* = 9.4, 2.1 Hz, 1H), 8.20 (s, 1H), 7.64 (d, *J* = 1.9 Hz, 1H), 7.30 (dd, *J* = 8.2, 1.9 Hz, 1H), 7.22 (d, *J* = 9.4 Hz, 1H), 6.90 (d, *J* = 8.2 Hz, 1H), 3.98 (s, 3H), 3.77 – 3.67 (m, 8H), 3.66 – 3.60 (m, 2H), 3.14 (t, *J* = 5.0 Hz, 2H). (97% yield)

HRMS [+ Scan]; calculated m/z for $C_{20}H_{28}N_5O_5$ 418.2085; observed mass 418.2073.



(E)-N-(2-(2-(2-aminoethoxy)ethoxy)ethyl)-6-(2-(2,4,6-trimethoxybenzylidene)hydrazinyl)nicotinamide (**2b-4**)

^{1}H NMR (500 MHz, Methanol-d_4) δ 8.60 (s, 1H), 8.50 (dt, *J* = 2.1, 1.1 Hz, 1H), 8.35 (dd, *J* = 9.4, 2.1 Hz, 1H), 7.17 (d, *J* = 9.4 Hz, 1H), 6.34 (s, 2H), 3.94 (s, 6H), 3.91 (s, 3H), 3.76 – 3.67 (m, 10H), 3.64 – 3.58 (m, 2H), 3.14 (t, *J* = 5.0 Hz, 2H). (75% yield)
HRMS [+ Scan]; calculated m/z for $C_{22}H_{32}N_5O_6$ 462.2347; observed mass 462.2340.



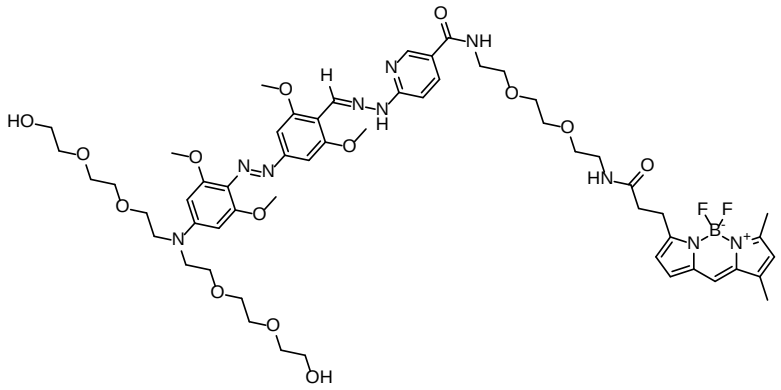
(E)-N-(2-(2-(2-aminoethoxy)ethoxy)ethyl)-6-(2-(2,4-dihydroxy-6-methylbenzylidene)hydrazinyl)nicotinamide (**2b-5**)

^{1}H NMR (500 MHz, Methanol-d_4) δ 8.67 (s, 1H), 8.51 (d, *J* = 2.1 Hz, 1H), 8.30 (dd, *J* = 9.3, 2.0 Hz, 1H), 7.12 (d, *J* = 9.3 Hz, 1H), 6.28 (d, *J* = 2.3 Hz, 1H), 6.24 (d, *J* = 2.3 Hz, 1H), 3.75 – 3.68 (m, 8H), 3.61 (t, *J* = 5.7 Hz, 2H), 3.14 (t, *J* = 5.0 Hz, 2H), 2.43 (s, 3H). (80% yield)
HRMS [+ Scan]; calculated m/z for $C_{20}H_{28}N_5O_5$ 418.2085; observed mass 418.2077.

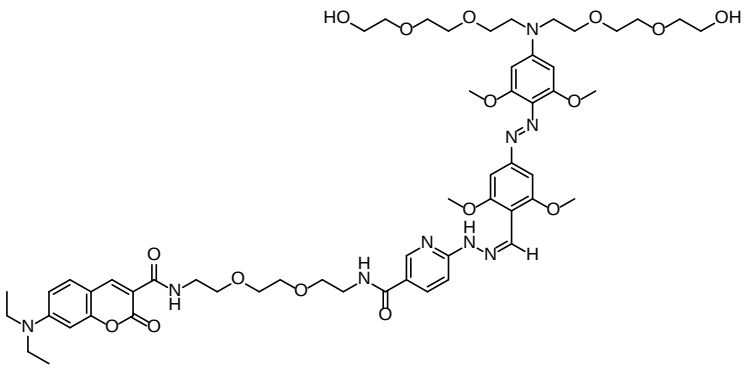
General procedure for fluorescent dye conjugation with 2b-6

2b-6 (1.8 mg 2.06 µmol) was added to a 1mL screwcap vial and 5-carboxyfluorescein NHS-ester (0.97 mg, 2.06 µmol) was predissolved in 0.2mL DMF and added to the reaction. Triethylamine (0.63 mg, 0.86 µL, 6.2 µmol) was added using a micropipettor and the reaction was stirred overnight with the protection of light.

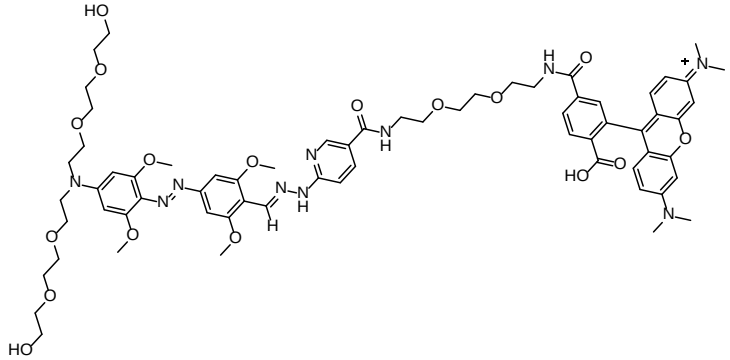
2 mL 3:7 acetonitrile: water was added and the reaction was filtered using a 0.22 µm filter and purified by RP-HPLC with a C18 column using water and acetonitrile with 0.1% TFA as eluent. The collected fractions were frozen and lyophilized to obtain the desired DarkZone dye.



55% yield. ^{1}H NMR (500 MHz, Methanol-d_4) δ 8.55 (s, 1H), 8.44 (s, 1H), 8.32 – 8.25 (m, 1H), 7.34 (s, 1H), 7.14 (s, 2H), 6.94 (d, J = 4.0 Hz, 1H), 6.53 (s, 1H), 6.48 (s, 1H), 6.28 (d, J = 4.0 Hz, 1H), 6.16 (s, 1H), 5.52 (s, 1H), 4.28 – 4.06 (m, 10H), 4.01 (s, 6H), 3.92 – 3.89 (m, 4H), 3.73 – 3.41 (m, 30H), 2.62 (t, J = 7.6 Hz, 2H), 2.47 (s, 3H), 2.25 (s, 3H).
HRMS [+ Scan]; calculated m/z for $C_{55}H_{76}BF_2N_{10}O_{14}$ 1149.5598; observed mass 1149.5614.

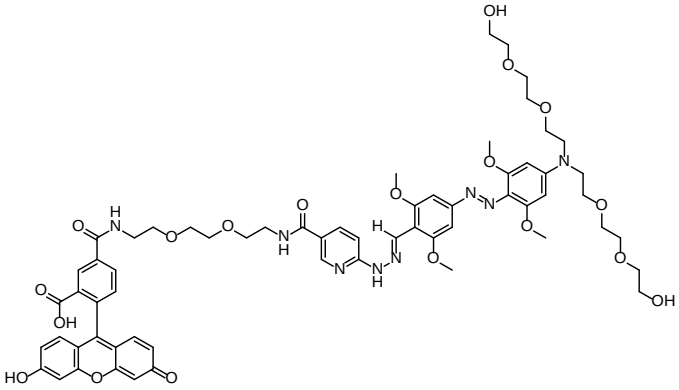


64% yield. ^{1}H NMR (500 MHz, Methanol-d_4) δ 8.61 (s, 1H), 8.58 (d, J = 2.1 Hz, 1H), 8.46 (s, 1H), 8.33 (d, J = 9.4 Hz, 1H), 7.44 (d, J = 9.0 Hz, 1H), 7.20 – 7.14 (m, 1H), 7.04 (s, 2H), 6.72 (dd, J = 9.0, 2.4 Hz, 1H), 6.56 – 6.45 (m, 2H), 6.41 (d, J = 2.3 Hz, 1H), 4.30 – 4.07 (m, 10H), 3.96 (d, J = 4.8 Hz, 6H), 3.90 (t, J = 4.9 Hz, 4H), 3.75 – 3.58 (m, 24H), 3.58 – 3.52 (m, 4H), 3.51 – 3.42 (m, 4H), 1.20 (t, J = 7.1 Hz, 6H).
HRMS [+ Scan]; calculated m/z for $C_{55}H_{76}N_9O_{16}$ 1118.5405; observed mass 1118.5421.

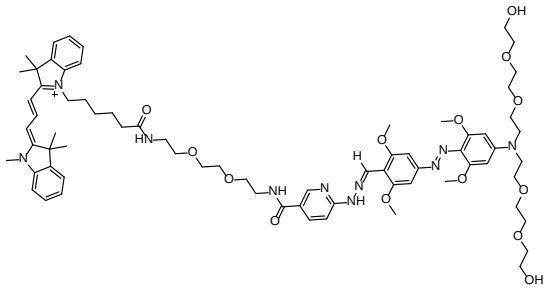


91% yield. ^{1}H NMR (500 MHz, Methanol-d_4) δ 8.42 – 8.36 (m, 2H), 8.35 (s, 1H), 8.26 (d, J = 8.5 Hz, 1H), 8.19 (s, 1H), 7.08 – 7.02 (m, 1H), 6.98 – 6.91 (m, 4H), 6.87 – 6.81

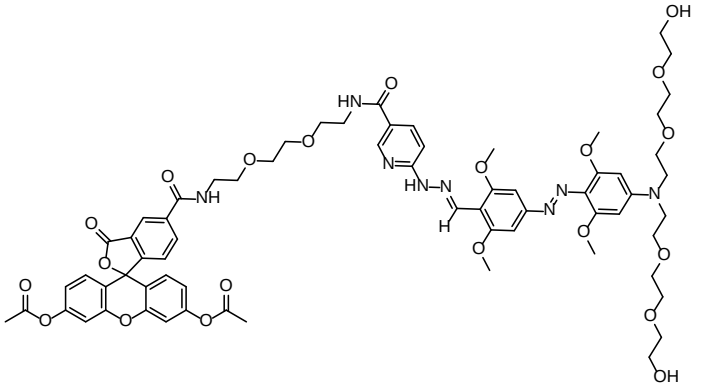
(m, 4H), 6.52 (s, 2H), 4.30 – 4.05 (m, 12H), 3.96 (s, 6H), 3.91 (t, *J* = 4.8 Hz, 4H), 3.86 – 3.50 (m, 26H), 3.25 (s, 12H).
HRMS [+ Scan]; calculated m/z for $C_{66}H_{83}N_{10}O_{17}$ 1287.5932; observed mass 1287.5946.



40% yield. ^{1}H NMR (500 MHz, Methanol-d_4) δ 8.57 (d, *J* = 2.1 Hz, 1H), 8.44 (s, 1H), 8.37 (s, 1H), 8.26 – 8.14 (m, 2H), 7.31 (d, *J* = 8.1 Hz, 1H), 7.23 (t, *J* = 8.8 Hz, 1H), 7.04 (s, 2H), 6.66 (d, *J* = 2.3 Hz, 2H), 6.57 – 6.38 (m, 6H), 4.29 – 4.01 (m, 12H), 3.96 (s, 6H), 3.94 – 3.86 (m, 4H), 3.79 – 3.50 (m, 26H).
HRMS [+ Scan]; calculated m/z for $C_{62}H_{73}N_8O_{19}$ 1233.4986; observed mass 1233.4991.

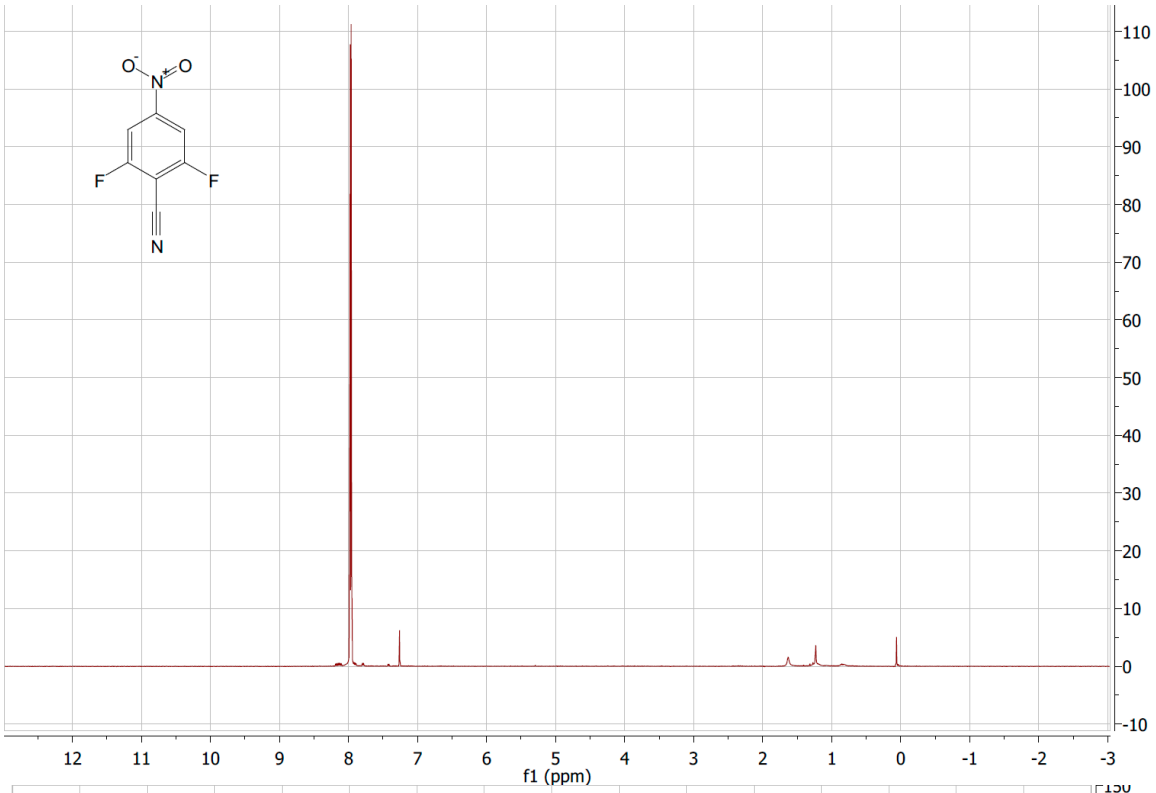


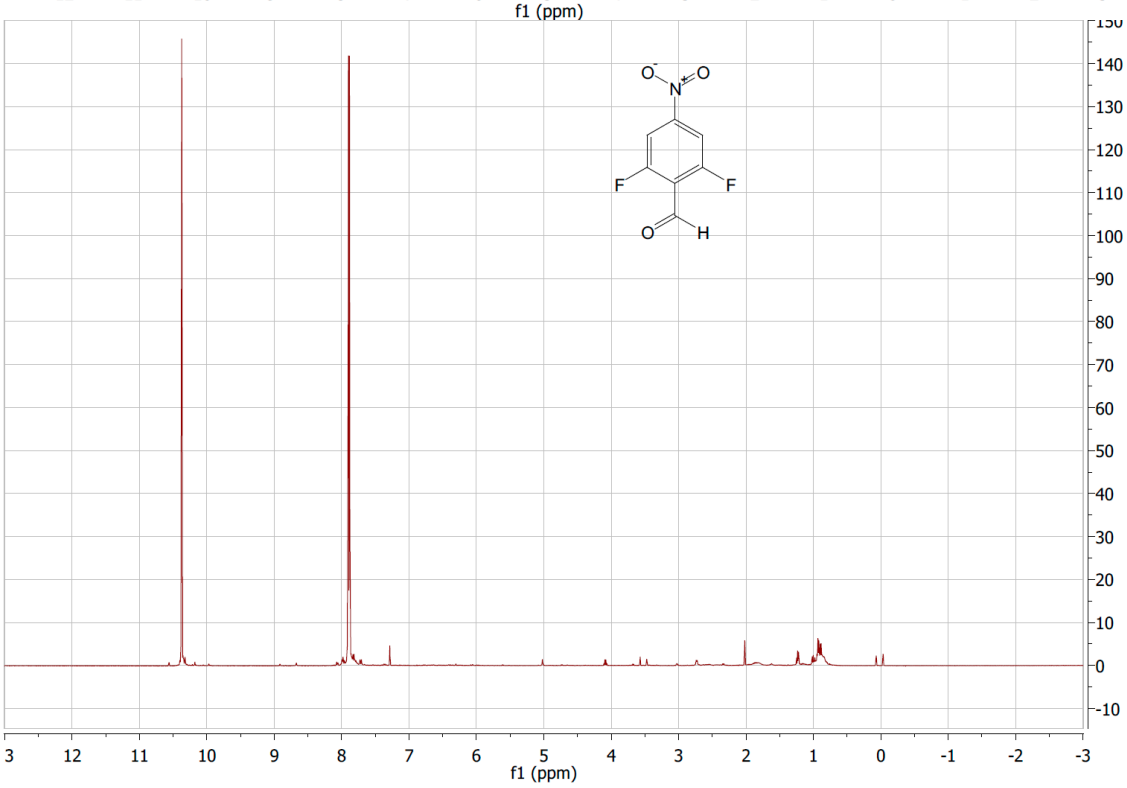
90% yield. ^{1}H NMR (500 MHz, Methanol-d_4) δ 8.57 (d, *J* = 1.8 Hz, 1H), 8.50 (t, *J* = 13.4 Hz, 1H), 8.42 (s, 1H), 8.16 (d, *J* = 9.0 Hz, 1H), 7.57 – 7.51 (m, 2H), 7.48 – 7.39 (m, 2H), 7.37 – 7.22 (m, 5H), 7.10 (s, 2H), 6.50 (s, 1H), 6.45 (s, 1H), 6.41 – 6.32 (m, 2H), 4.27 – 4.05 (m, 12H), 3.98 (s, 6H), 3.90 (t, *J* = 5.0 Hz, 4H), 3.75 – 3.51 (m, 28H), 3.39 – 3.35 (m, 2H), 2.23 (t, *J* = 7.3 Hz, 2H), 1.89 – 1.66 (m, 16H), 1.48 (d, *J* = 7.8 Hz, 2H).
HRMS [+ Scan]; calculated m/z for $C_{71}H_{97}N_{10}O_{14}$ 1313.7180; observed mass 1313.7181.

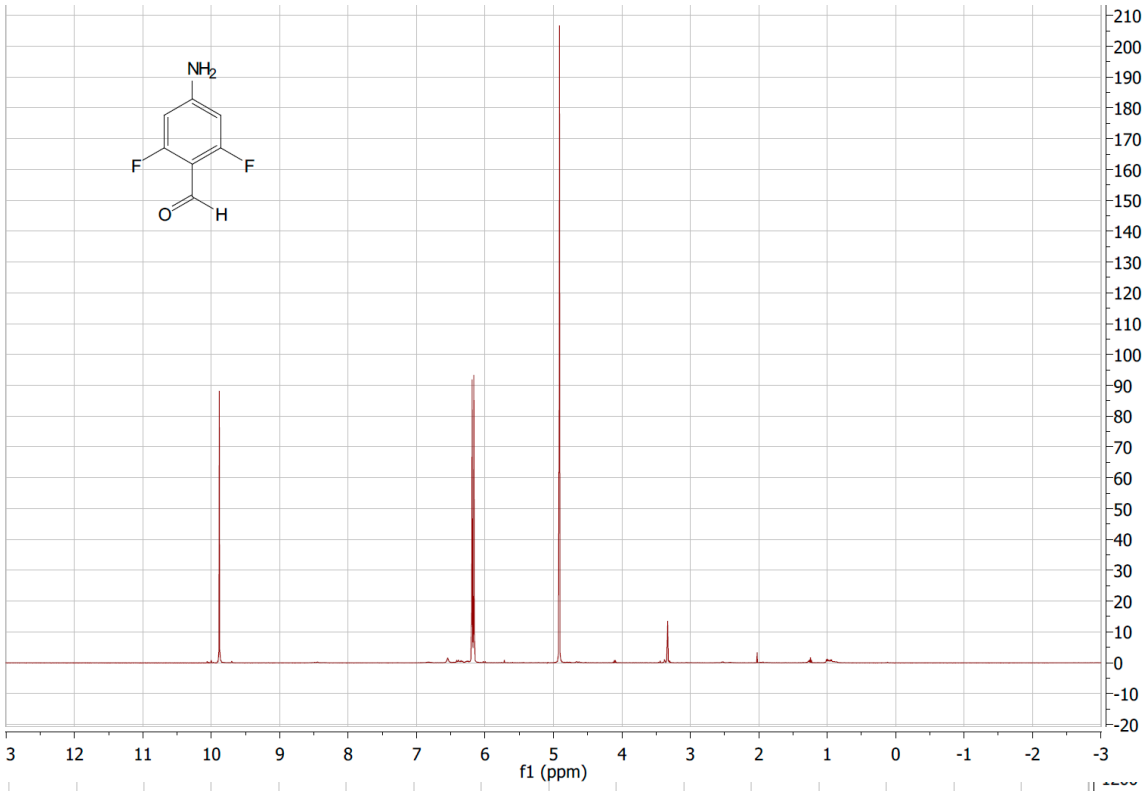


^{1}H NMR (500 MHz, Methanol-d_4) δ 8.55 (dd, J = 2.2, 0.8 Hz, 1H), 8.46 (dd, J = 1.6, 0.8 Hz, 1H), 8.34 (s, 1H), 8.23 (dd, J = 8.1, 1.6 Hz, 1H), 7.37 (d, J = 8.1 Hz, 1H), 7.20 (d, J = 9.0 Hz, 1H), 7.15 (d, J = 2.3 Hz, 2H), 7.02 (d, J = 4.1 Hz, 2H), 6.88 (d, J = 2.3 Hz, 1H), 6.87 (d, J = 2.2 Hz, 1H), 6.82 (s, 1H), 6.80 (s, 1H), 6.51 – 6.41 (m, 2H), 4.28 – 4.03 (m, 12H), 3.95 (s, 6H), 3.92 – 3.85 (m, 4H), 3.78 – 3.52 (m, 26H), 2.30 (s, 6H).
HRMS [+ Scan]; calculated m/z for $C_{66}H_{77}N_8O_{21}$ 1317.5198; observed mass 1317.5200.

^{1}H NMR



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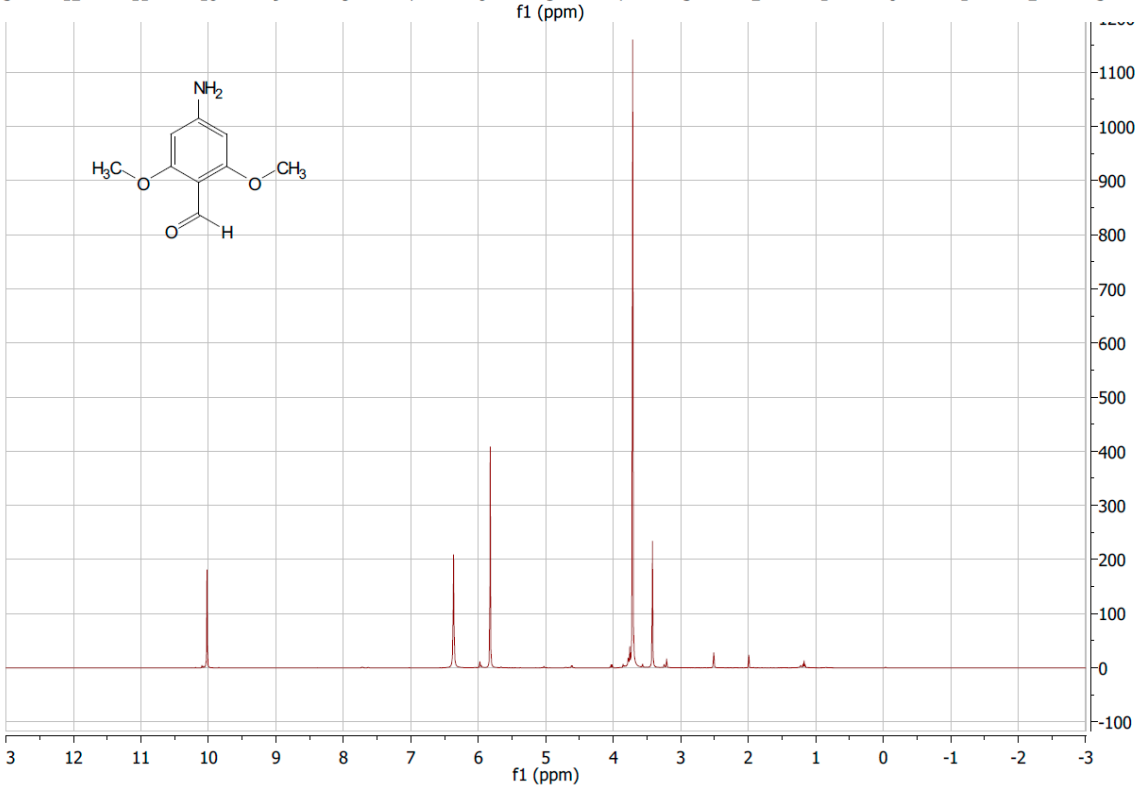
F
O
H
f1 (ppm)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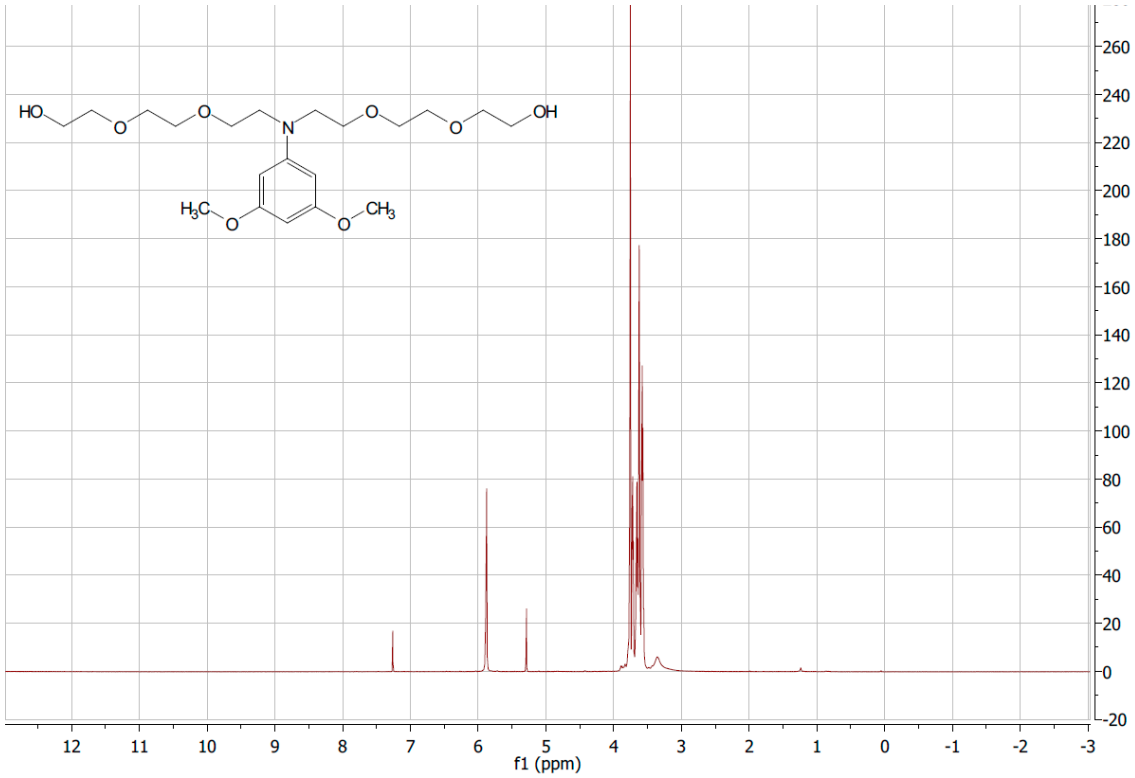
O
O
CH3
O
H
f1 (ppm)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OH
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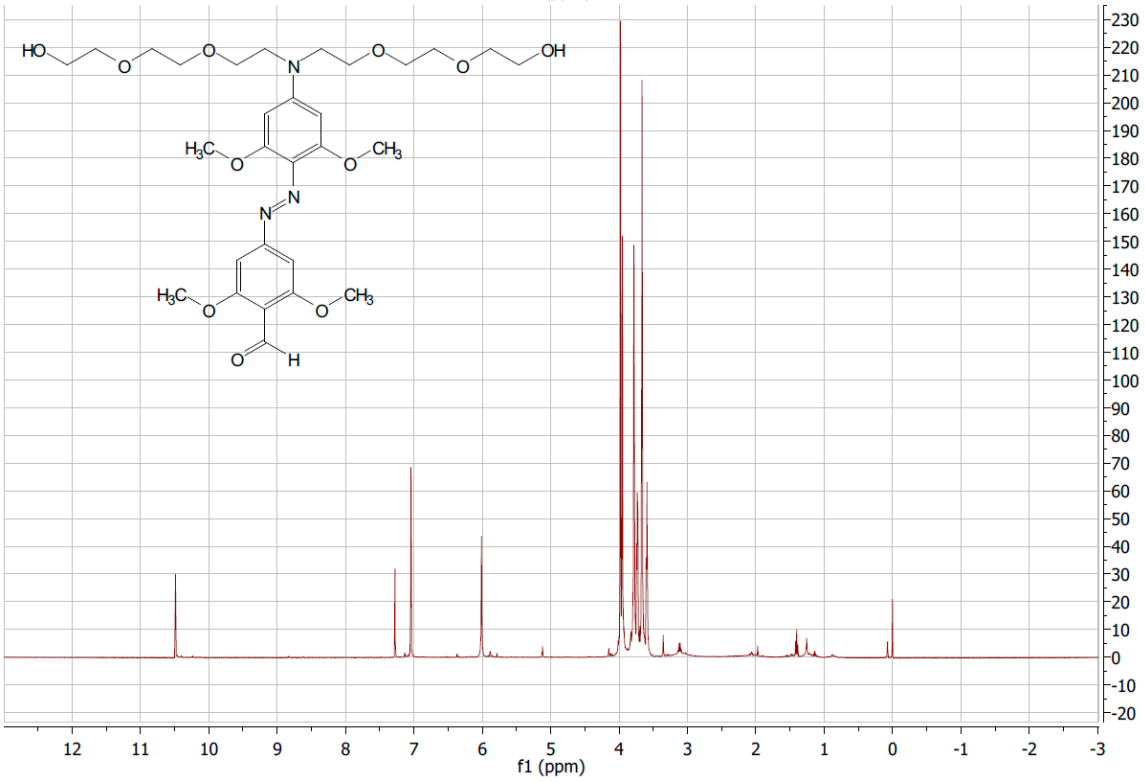
O
O
CH3
f1 (ppm)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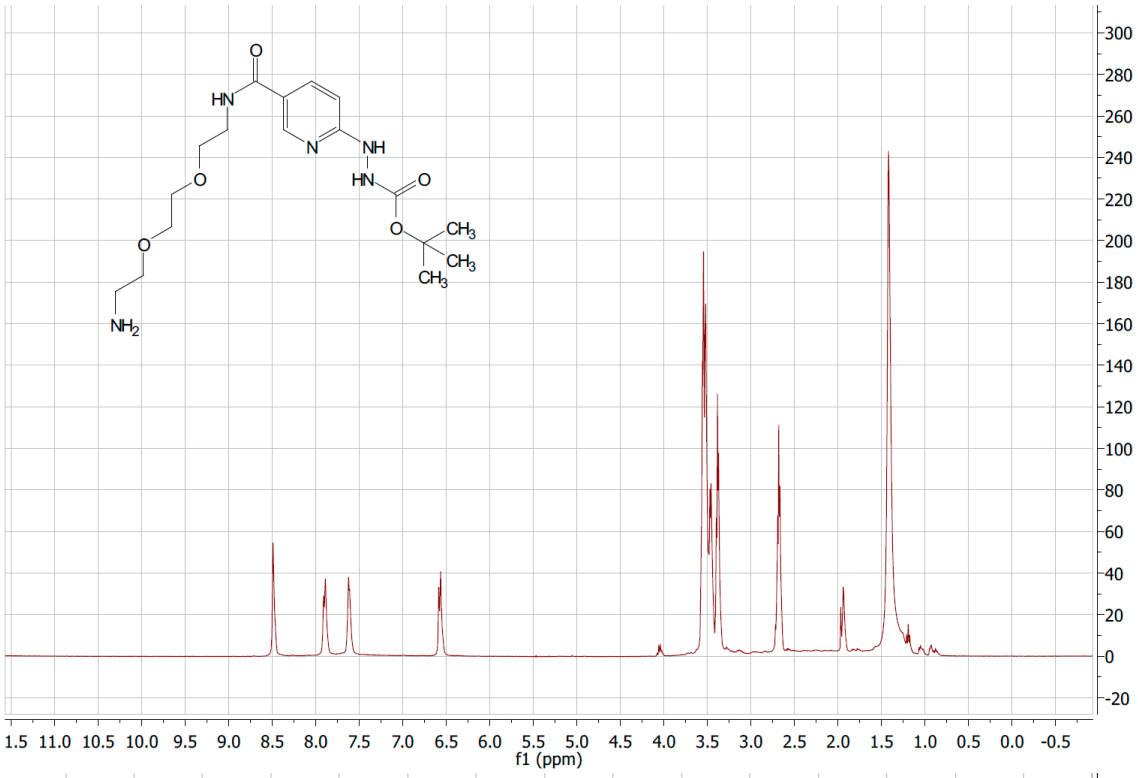
f1 (ppm)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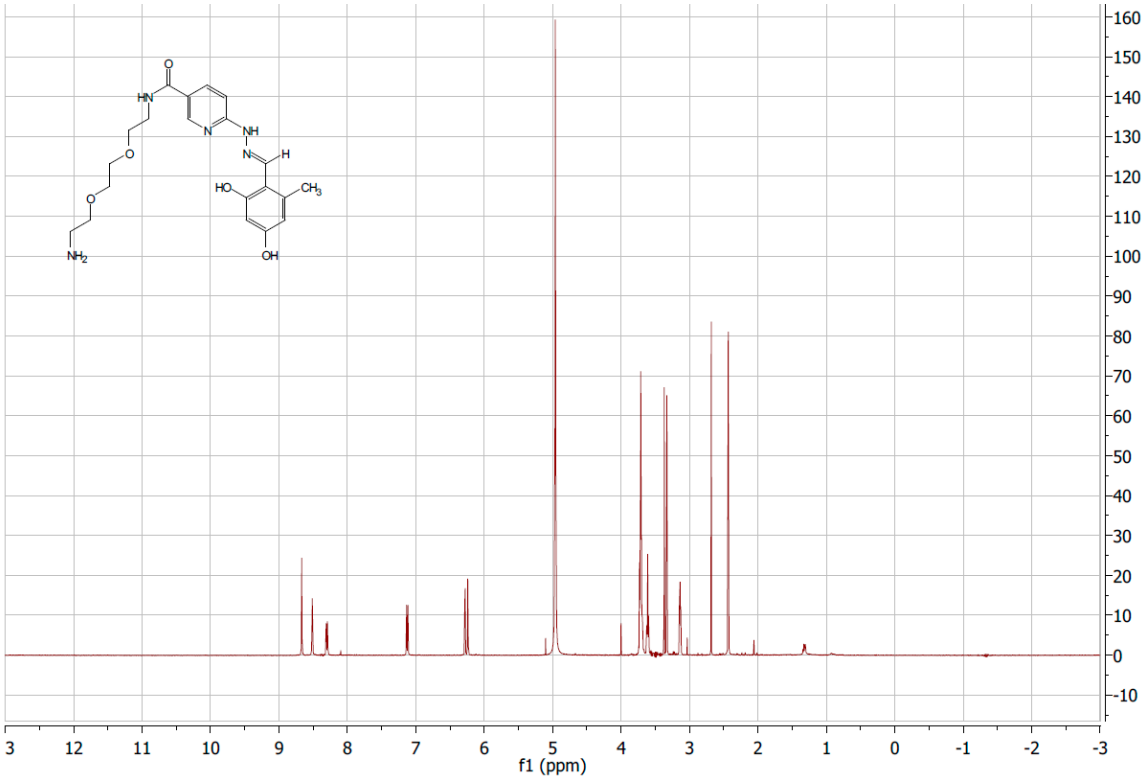
NH
HN
O
O
O
O
CH3
CH3
CH3
NH2
f1 (ppm)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NH
N
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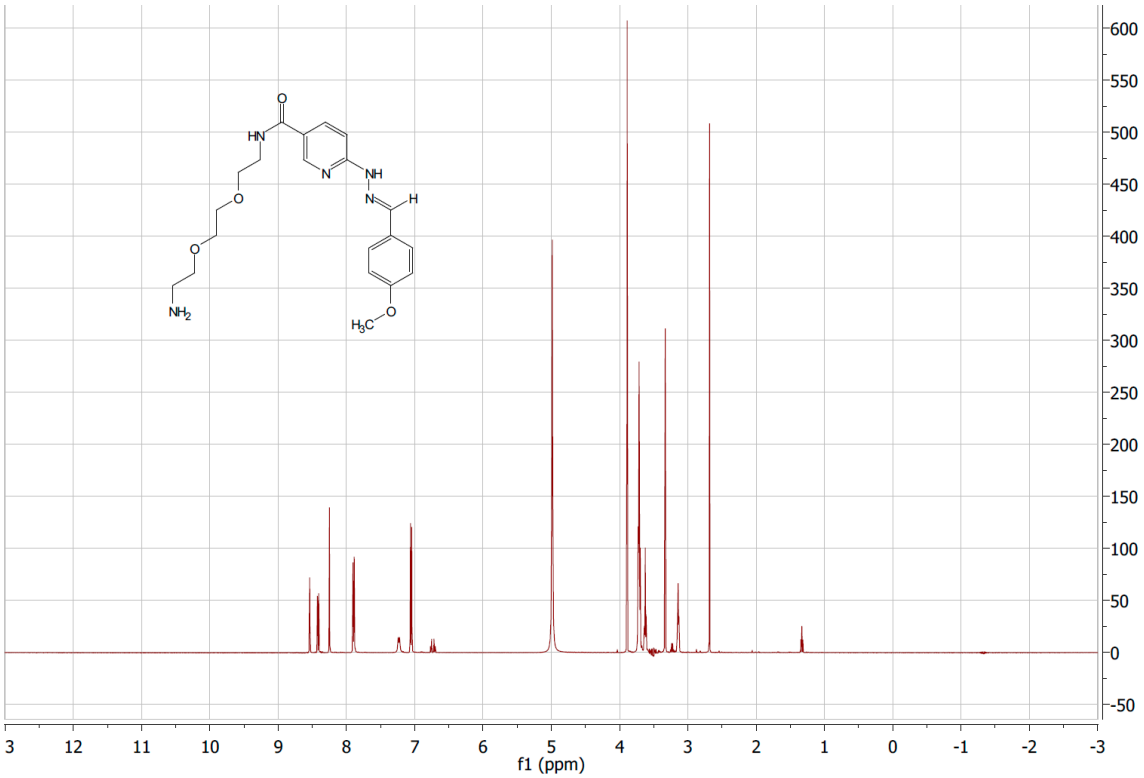
CH3
OH
O
O
NH2
f1 (ppm)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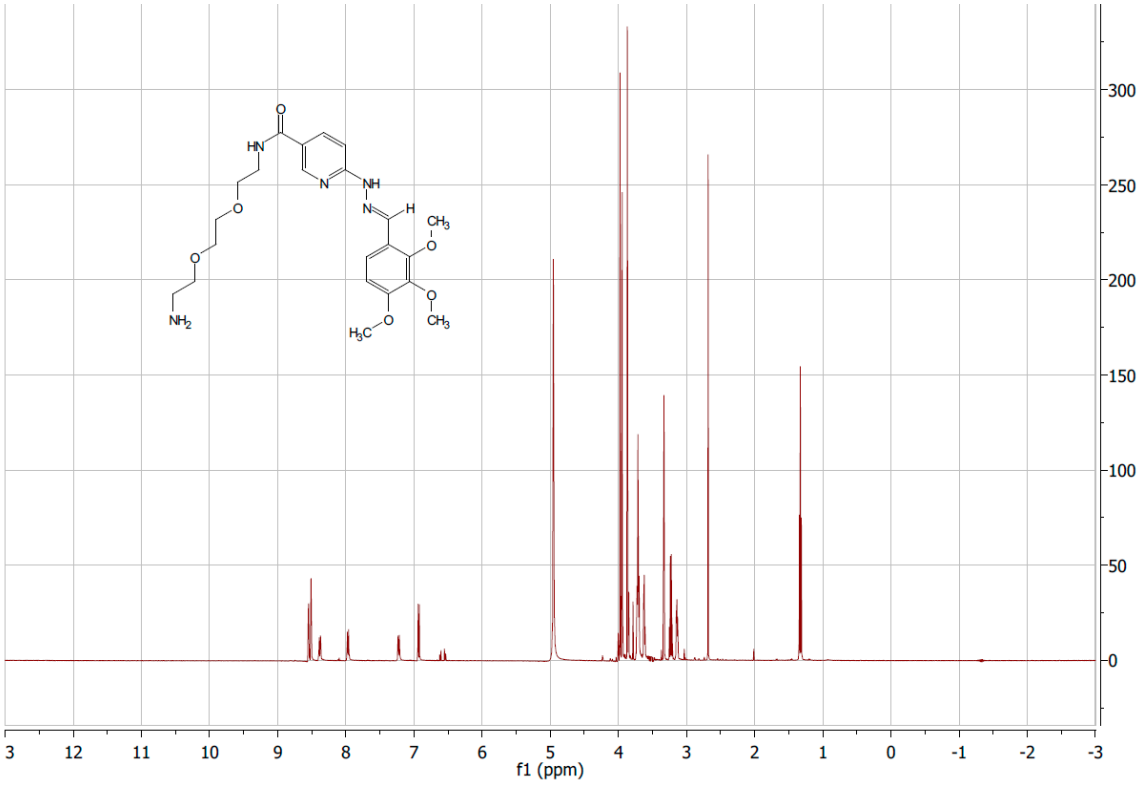
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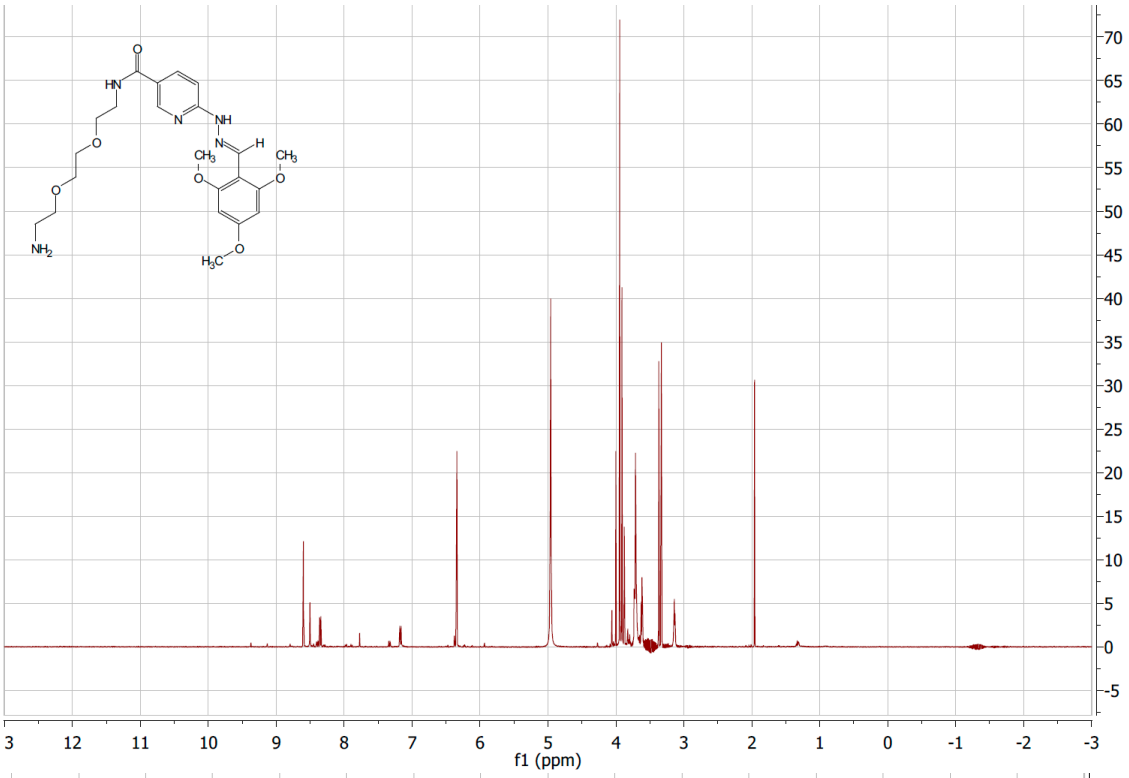
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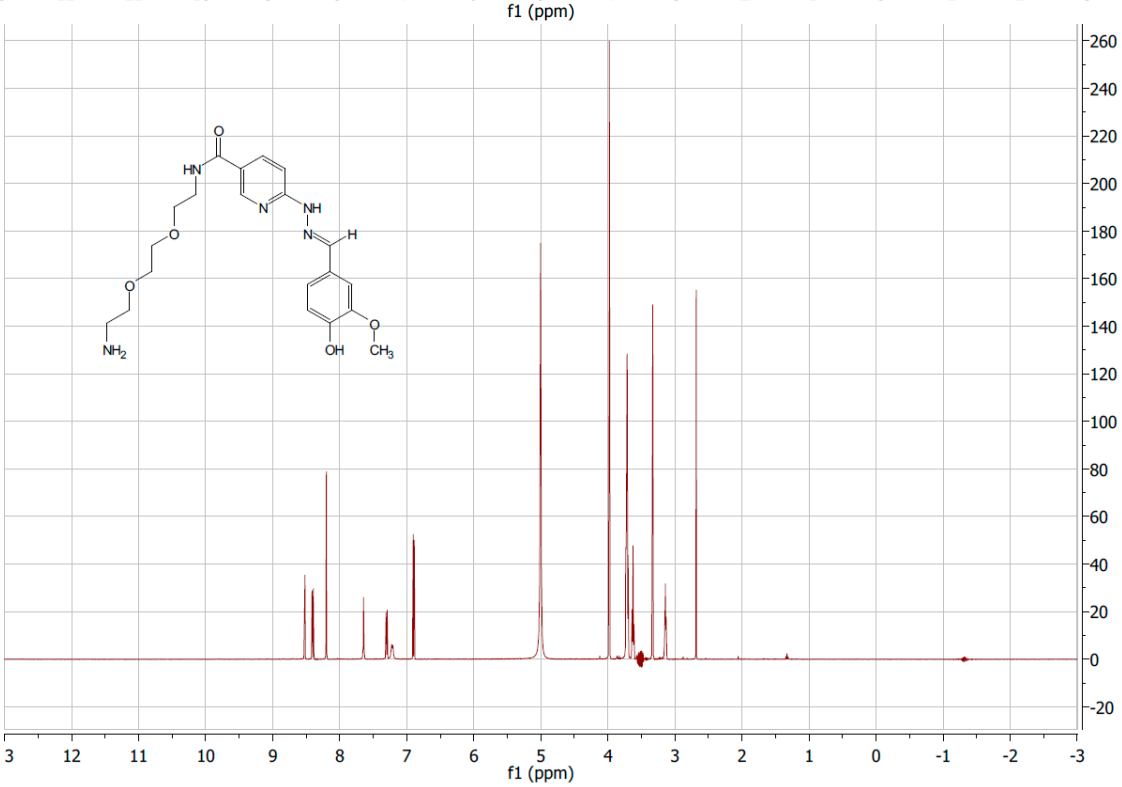
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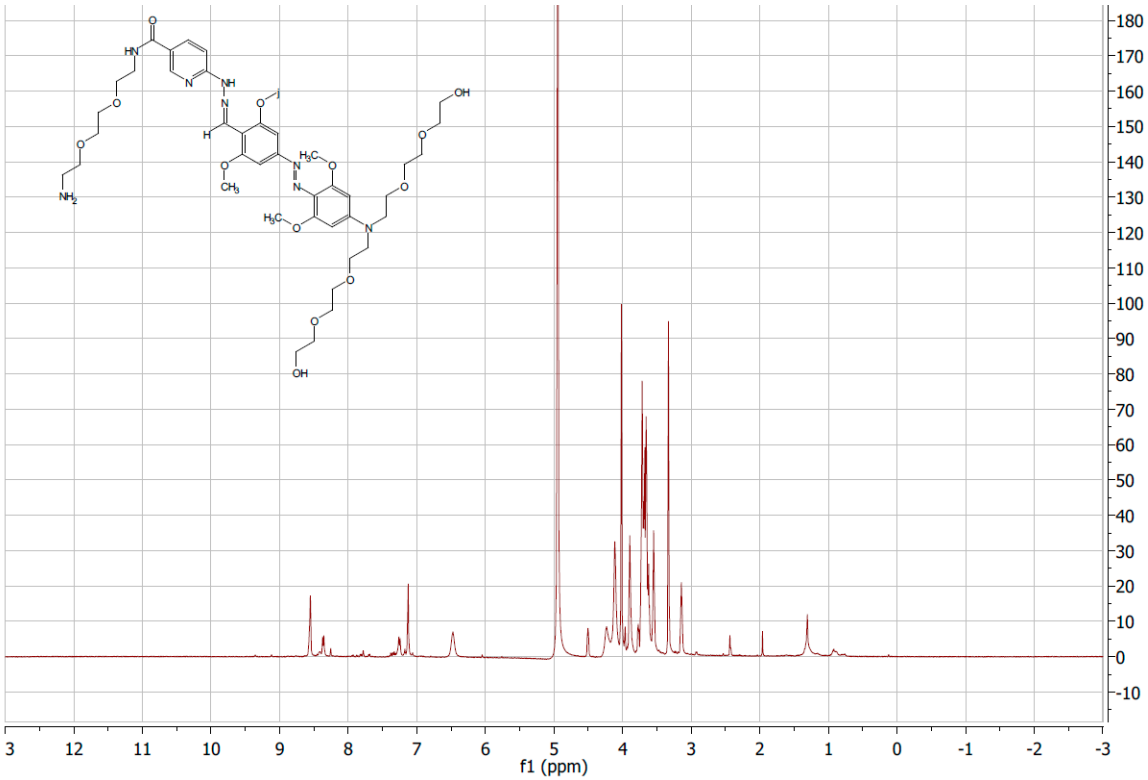
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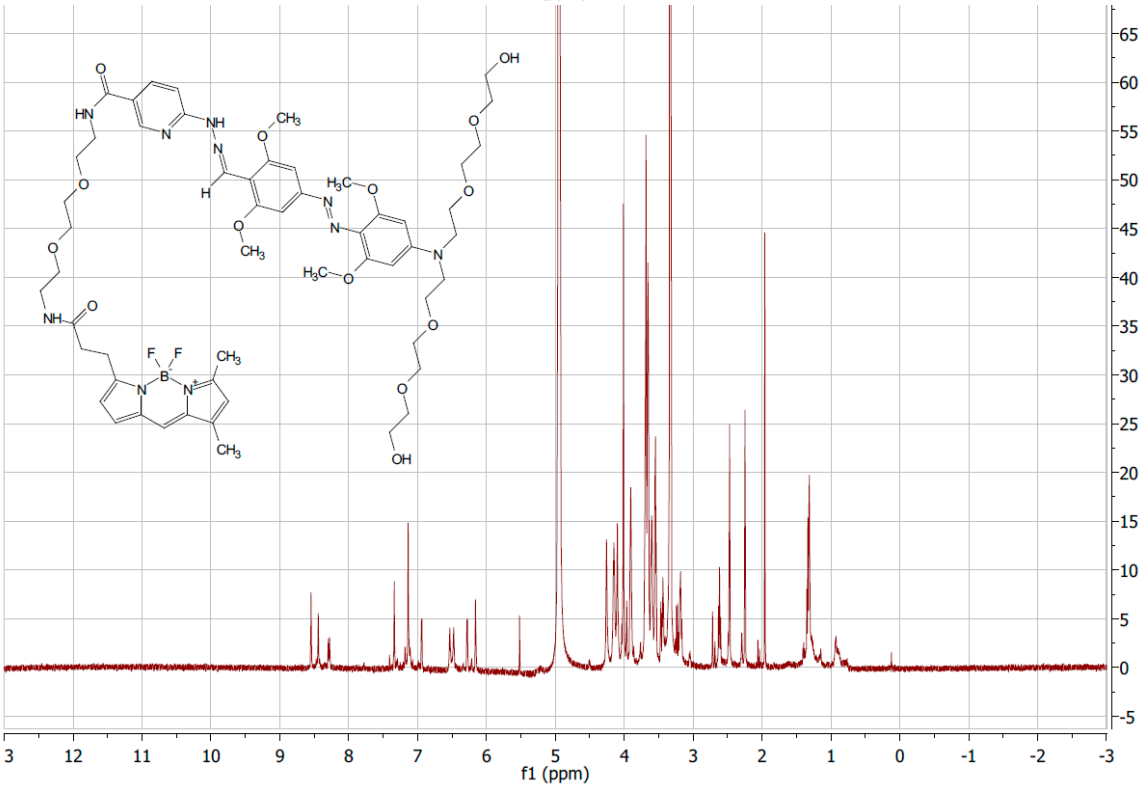
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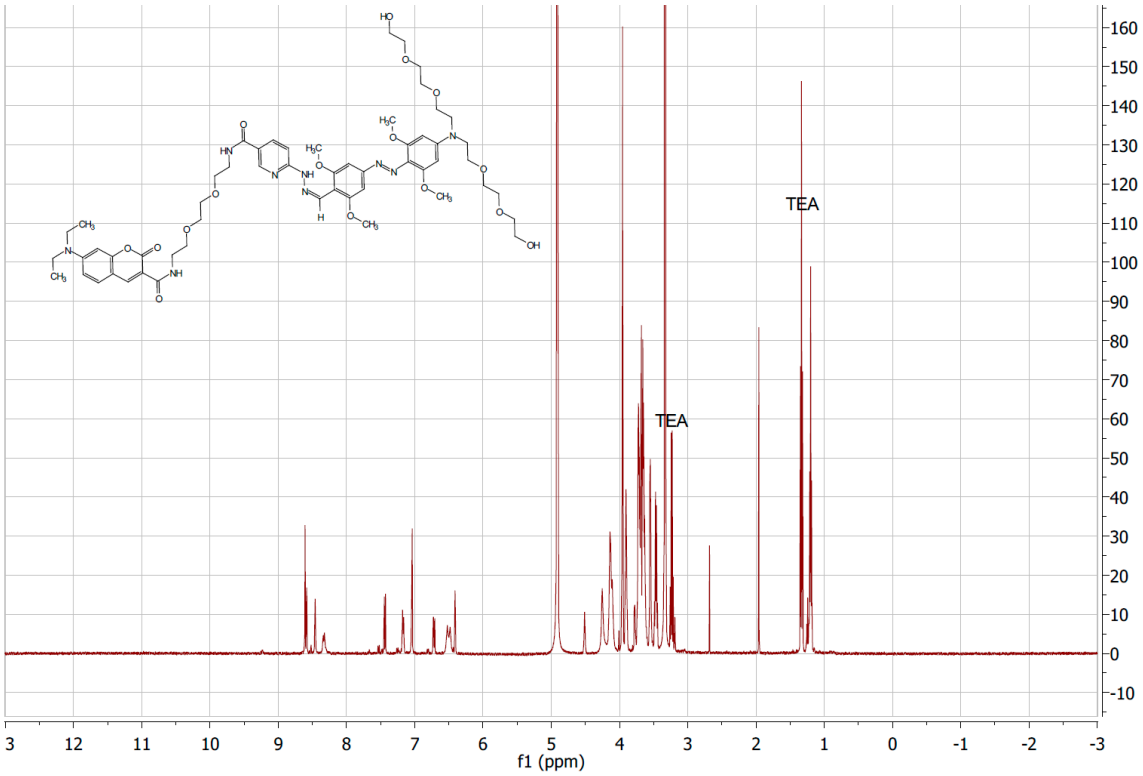
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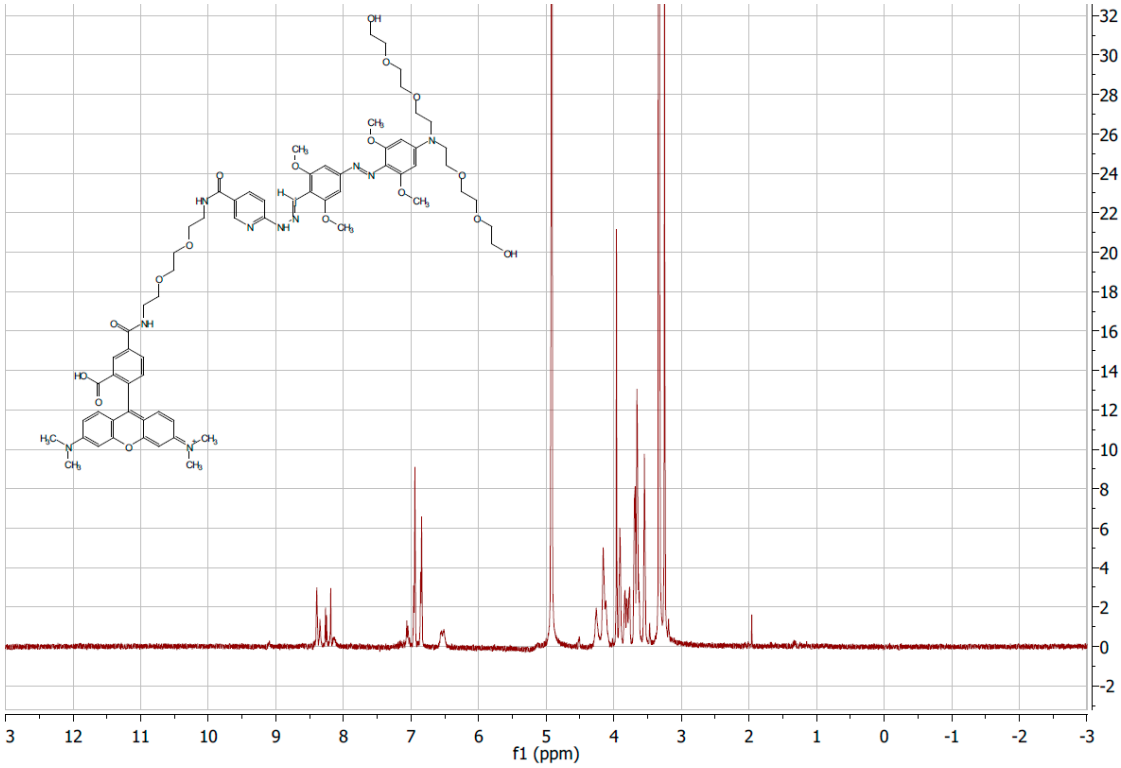
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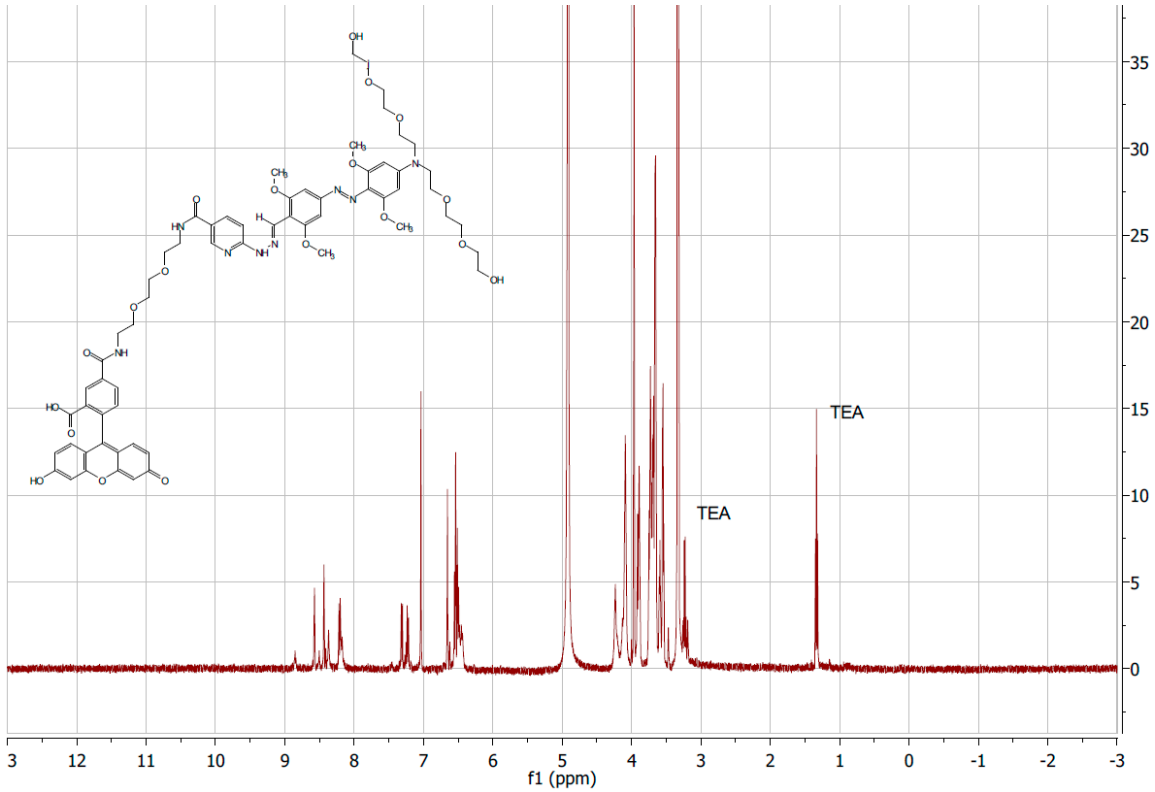
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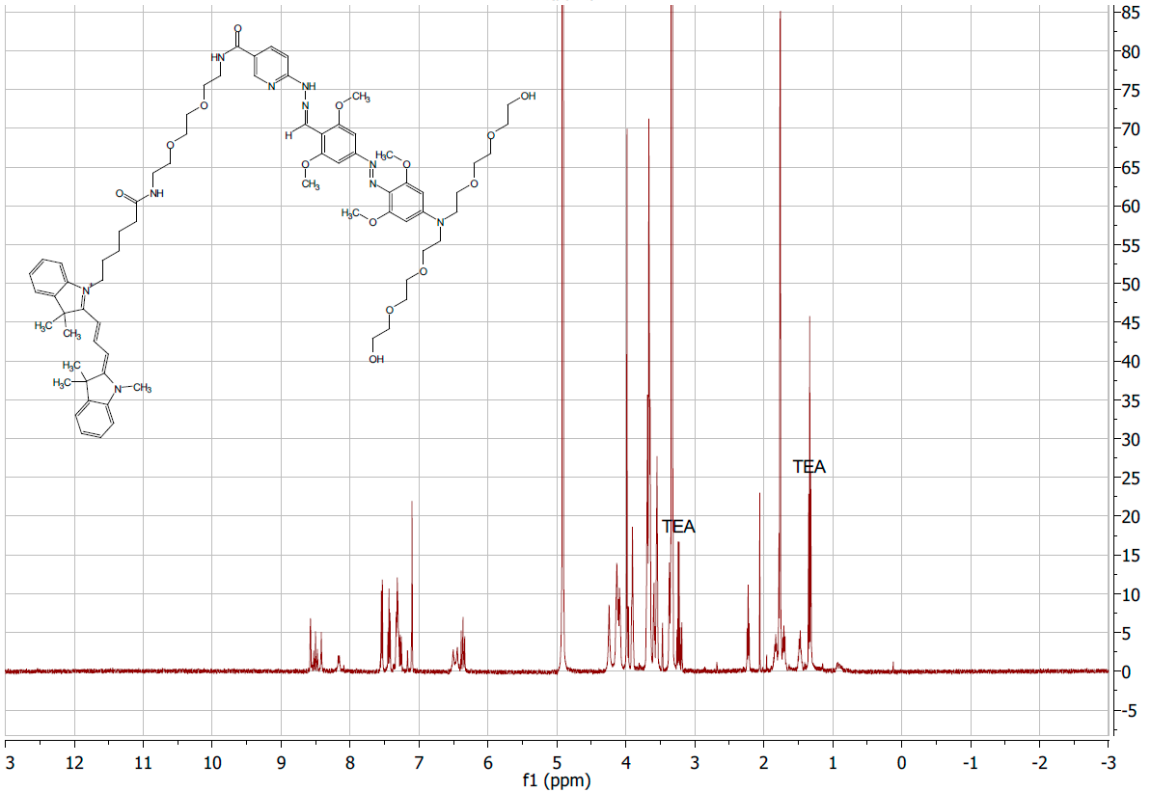
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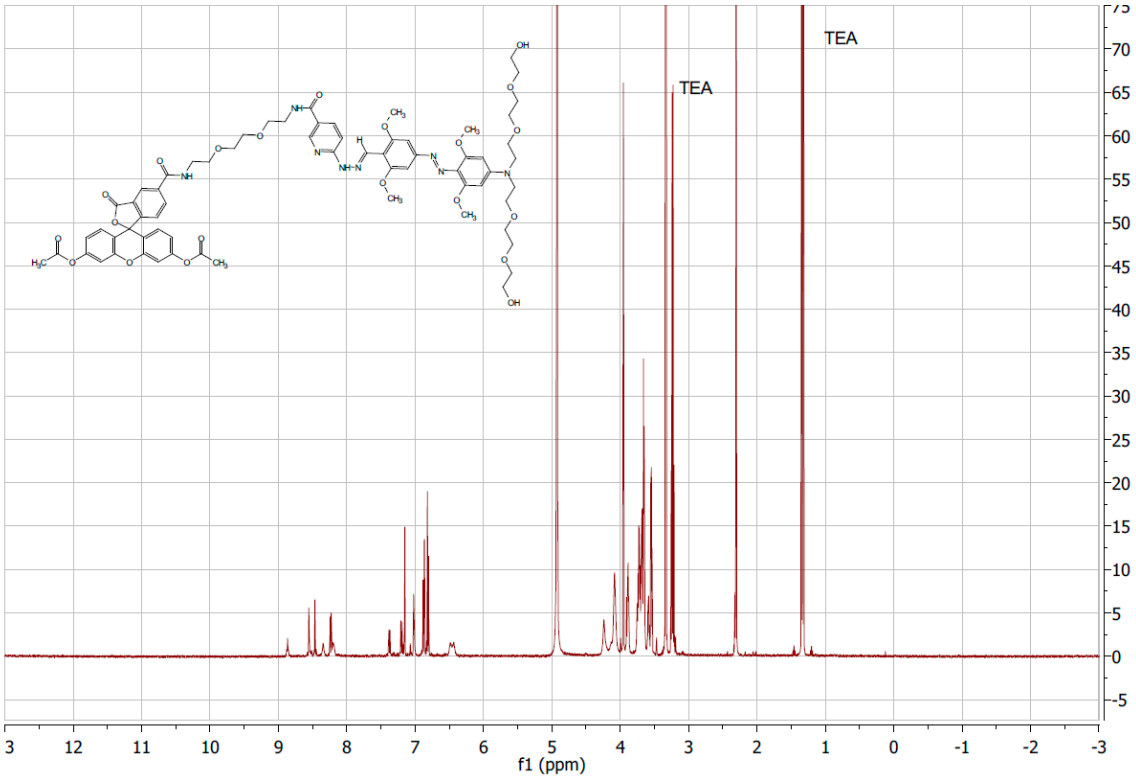
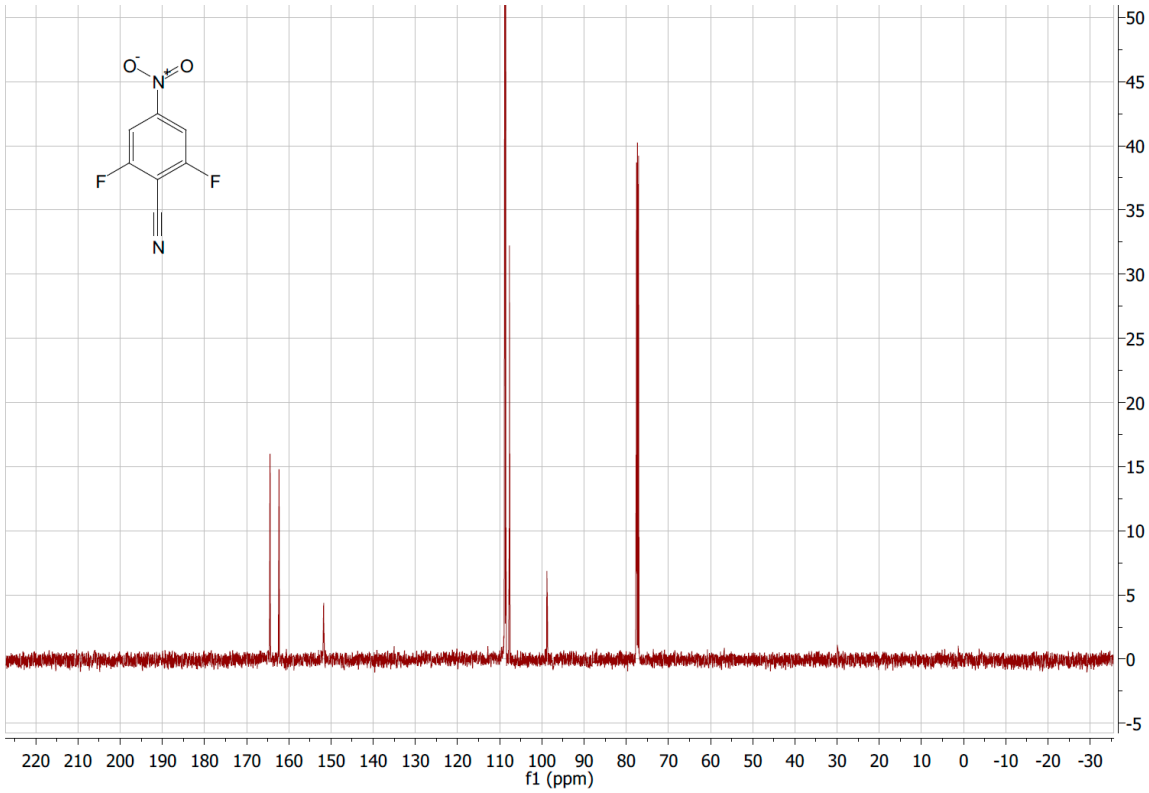
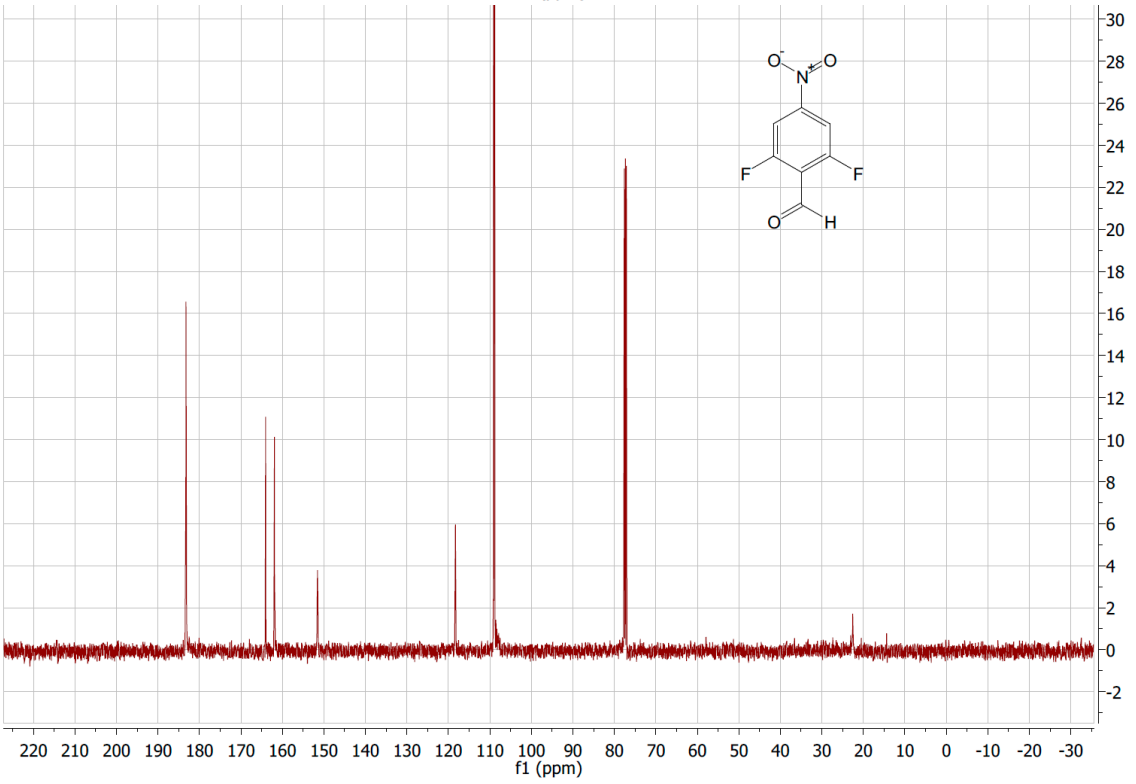
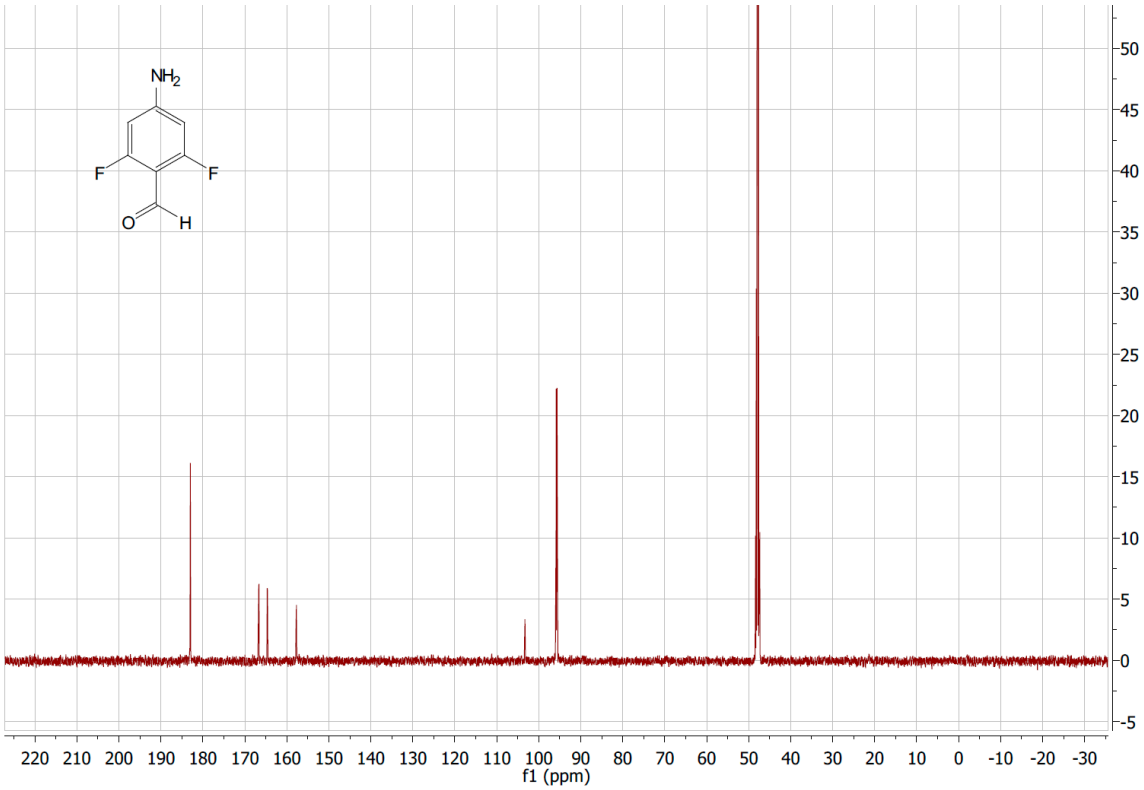
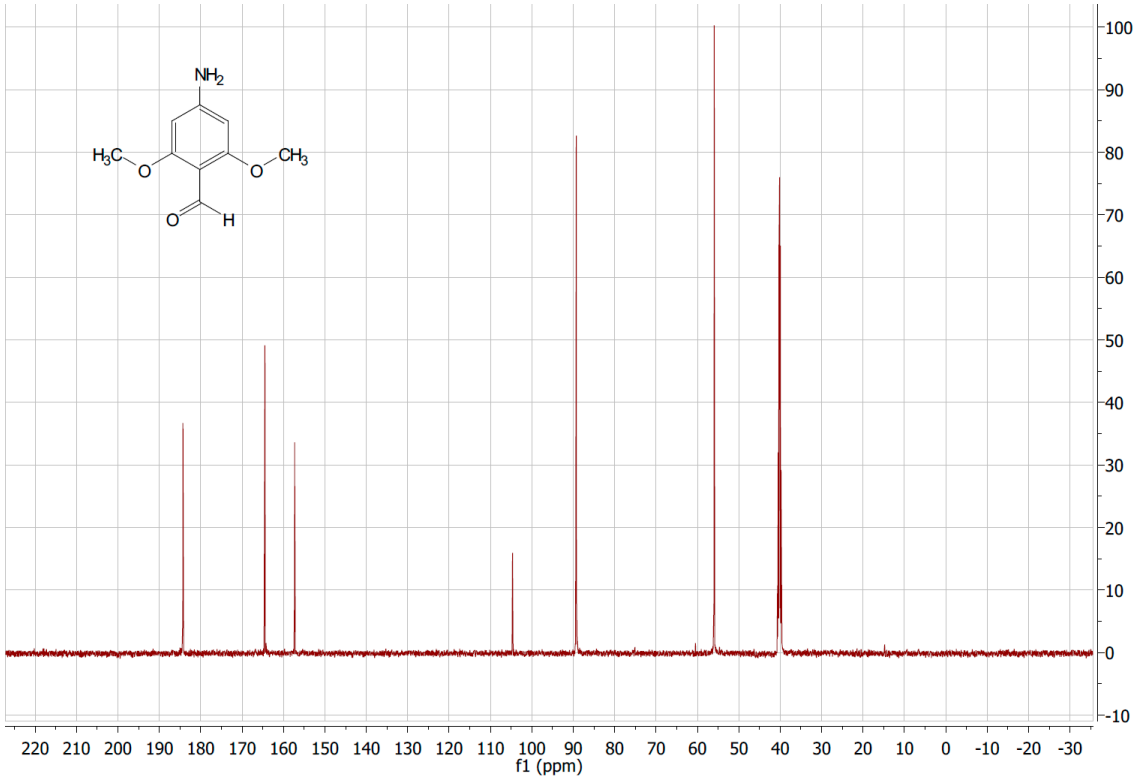

TEA
TEA
f1 (ppm)

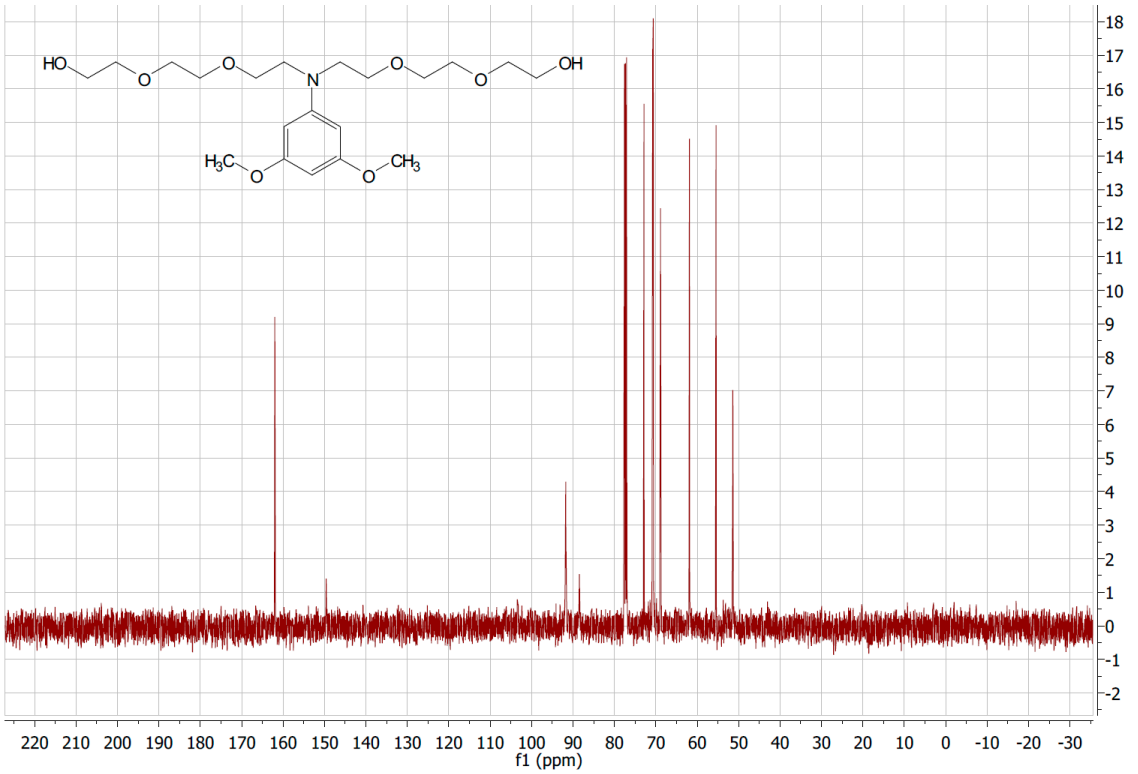
^{13}C NMR

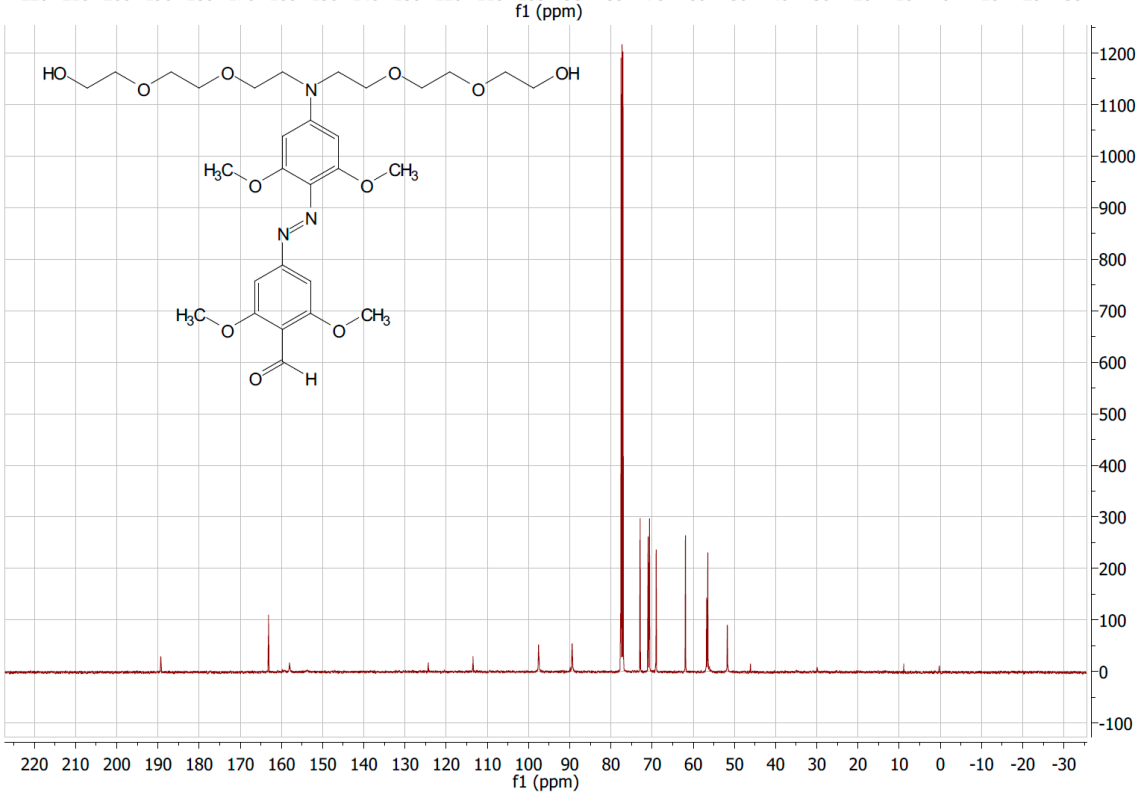

O
N
O
F
F
N
220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10 -20 -30
f1 (ppm)
50 45 40 35 30 25 20 15 10 5 0 -5

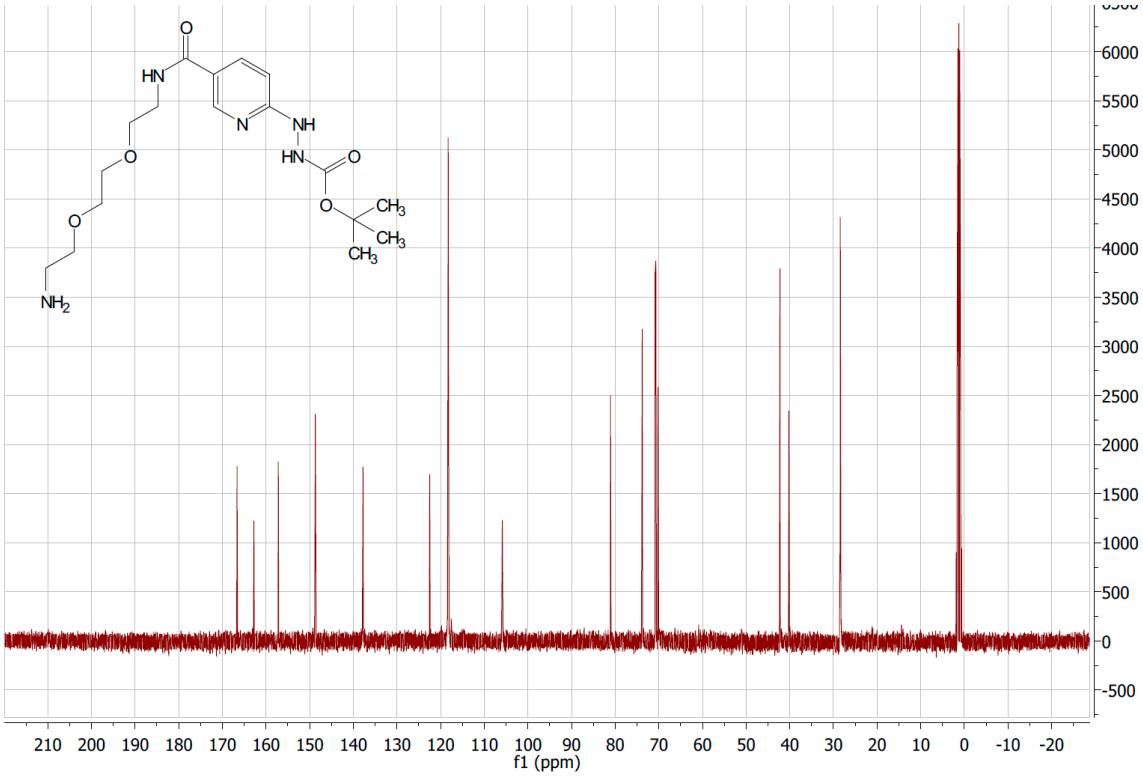

O
N
O
F
F
O
H
220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10 -20 -30
f1 (ppm)
30 28 26 24 22 20 18 16 14 12 10 8 6 4 2 0 -2


NH2
F
F
O
H
50
45
40
35
30
25
20
15
10
5
0
-5
220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10 -20 -30
f1 (ppm)


NH2
H3C
O
O
CH3
O
H
100
90
80
70
60
50
40
30
20
10
0
-10
220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10 -20 -30
f1 (ppm)


HO
O
O
N
O
O
OH
H3C
O
O
CH3
18 17 16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1 0 -1 -2
220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10 -20 -30
f1 (ppm)


HO
O
O
N
O
O
OH
H3C
O
O
CH3
N=N
H3C
O
O
CH3
O
H
1200 1100 1000 900 800 700 600 500 400 300 200 100 0 -100
220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10 -20 -30
f1 (ppm)



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