Supporting Information for

Development of a General Aza-Cope Reaction Trigger Applied to Fluorescence Imaging of Formaldehyde in Living Cells

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Selectivity Tests. A 10 μM solution of **probe** in 20 mM PBS (pH 7.4) was prepared by diluting a 10 mM DMSO stock solution of probe into pre-warmed PBS (37 °C) in a 1-cm × 1-cm quartz cuvette. The analyte of interest was added to the cuvette to bring the concentration to 100 μM (unless otherwise specified), vigorously mixed with a pipette, and then a t=0 timepoint was collected. The cuvette was incubated in a 37 °C water bath throughout the course of the experiment. Emission spectra were taken at t = 0, 20, 45, 60, 90, and 120 min.

FA: 10 μL of a 10 mM stock solution in Milli-Q water (prepared on the same day by diluting 7.5 μL commercial wt. 37% FA solution with 1 mL Milli-Q water, followed by another dilution of 100 μL of the resulting solution in 900 μL Milli-Q water) was added to 990 μL of 10 μM solution of probe in PBS.

Acetaldehyde: 10 μL of a 10 mM stock solution in Milli-Q water (prepared on the same day by diluting 2.8 μL acetaldehyde with 5 mL Milli-Q water) was added to 990 μL of 10 μM solution of probe in PBS.

Glucose: 10 μL of a 1 M stock solution in Milli-Q water (prepared on the same day by dissolving 180 mg of glucose in 10 mL Milli-Q water) was added to 990 μL of 10 μM solution of probe in PBS.

4-hydroxynonenal (4-HNE): 1.56 μL of a commercial stock solution of 4-HNE in EtOH was added to 998.4 μL of 10 μM solution of probe in PBS.

Dehydroascorbate: 10 μL of a 10 mM stock solution in Milli-Q water (prepared on the same day by dissolving 0.87 mg dehydroascorbate with 500 μL of a 1:1 Milli-Q water/DMSO solution) was added to 990 μL of 10 μM solution of probe in PBS.

Glucosone: 10 μL of a 10 mM stock solution in Milli-Q water (prepared on the same day by dissolving 0.9 mg glucosone with 500 μL Milli-Q water) was added to 990 μL of 10 μM solution of probe in PBS.

Pyruvate: 10 μL of a 10 mM stock solution in Milli-Q water (prepared on the same day by dissolving 11 mg sodium pyruvate in with 10 mL Milli-Q water) was added to 990 μL of 10 μM solution of probe in PBS.

Oxaloacetate: 10 μL of a 10 mM stock solution in Milli-Q water (prepared on the same day by dissolving 15 mg oxaloacetic acid in 11.35 mL Milli-Q water) was added to 990 μL of 10 μM solution of probe in PBS.

Acrolein: 10 μL of a 10 mM stock solution in Milli-Q water (prepared on the same day by diluting 3.7 μL acrolein with 5 mL Milli-Q water) was added to 990 μL of 10 μM solution of probe in PBS.

Methylglyoxal: 10 µL of a 10 mM stock solution in Milli-Q water (prepared on the same day by diluting 7.7 µL methylglyoxal with 5 mL Milli-Q water) was added to 990 µL of 10 µM solution of probe in PBS.

Hydrogen peroxide: 10 µL of a 10 mM stock solution in Milli-Q water (prepared on the same day by diluting 10.2 µL commercial 9.8 M H_2O_2 with 10 mL Milli-Q water) was added to 990 µL of 10 µM solution of probe in PBS.

Glutathione: Prior to the experiment, all buffers and stocks were deoxygenated by bubbling a stream of nitrogen gas for 30 minutes. 50 µL of a 10 mM stock solution in PBS water (prepared on the same day by dissolving 615 mg glutathione with deoxygenated PBS, and then adjusting the pH by 1 M NaOH addition for a total volume of 20 mL) was added to 950 µL of 10 µM solution of probe in deoxygenated PBS. For the 2 h experiment, the reaction contents were capped from the air using a screw-top cuvette.

CRISPR/Cas9-mediated gene disruption of *ADH5* in HAP1 cells. Guide sequences (see **Table 1**) were cloned into pX461 vectors.[1] HAP1 cells were transfected with Turbofection 8.0 (Origene). Two days post-transfection, GFP-positive cells were single-cell sorted in 96-well plates containing medium supplemented with 20 % fetal calf serum, using a MoFlo cell sorter (Beckman-Coulter). After 14 days of incubation at 37 °C, individual clones were analyzed for expression of ADH5 by Western blotting. The targeted locus was subjected to Sanger sequencing (GATC). **Table 2** contains the primers used to amplify the locus by PCR.

Table 1: Guide sequences

Gene	Exon	Targeting sequence	Top oligo	Bottom oligo
ADH5	3_fwd[a]	CGCAGTGGCAATGATC TATCAGG	CACCGCGCAGTGGCAATGAT CTATC	AAACGATAGATCATTGCCAC TGCGC
ADH5	3_rev[a]	CCTGAGTGGAGCTGAT CCTGAGG	CACCGCTGAGTGGAGCTGAT CCTGA	AAACTCAGGATCAGCTCCAC TCAGC

Table 2: Primers used to amplify the locus

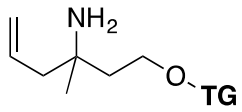
Gene	Exon	Fwd primer	Rev primer
ADH5	3	TGTTTTGCACACAGGCGATG	TGTGCTTCCCAGATGAGGAA

Western blotting. HAP1 cells were lysed for 30 min on ice in RIPA buffer (Thermo Fisher Scientific), including protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (Roche). Protein samples were run on a 4-12 % Bis-Tris gel (Thermo Fisher Scientific) and transferred to a 0.45 µm nitrocellulose membrane. Membranes

were blotted with antibodies against ADH5[2] and β-actin (Millipore, clone C4, MAB1501R).

Flow Cytometry. Flow cytometry experiments were performed with BD LSR Fortessa Cell Analyzer in the College of Chemistry at UC Berkeley or a BD LSR Fortessa X20 Cell Analyzer in the Flow Cytometry Facility at UC Berkeley. Unless otherwise stated, cells were plated in a 12-well polystyrene culture plate (Corning) and allowed to grow to the same confluence as in the corresponding confocal experiment. Gains were set to include live cells based on viability experiments using propidium iodide or Sytox Red exclusion (**Figure S14**). The standard filter set FITC-A (505 nm longpass, 530/30 nm bandpass) was used for **FAP488** and PE-A (570 nm longpass, 586/15 nm bandpass) for **FAP555** and **FAP573**. For each biological replicate, three technical replicates of 10,000 cells were counted for HEK293T experiments (**Figures S11-S13**) and three technical replicates of 5,000 cells were counted for HAP-1 experiments (**Figures S17 and S19**)**.** Flow cytometry data was processed using FlowJo (FlowJo, LLC).

Supplemental Figures



(6)

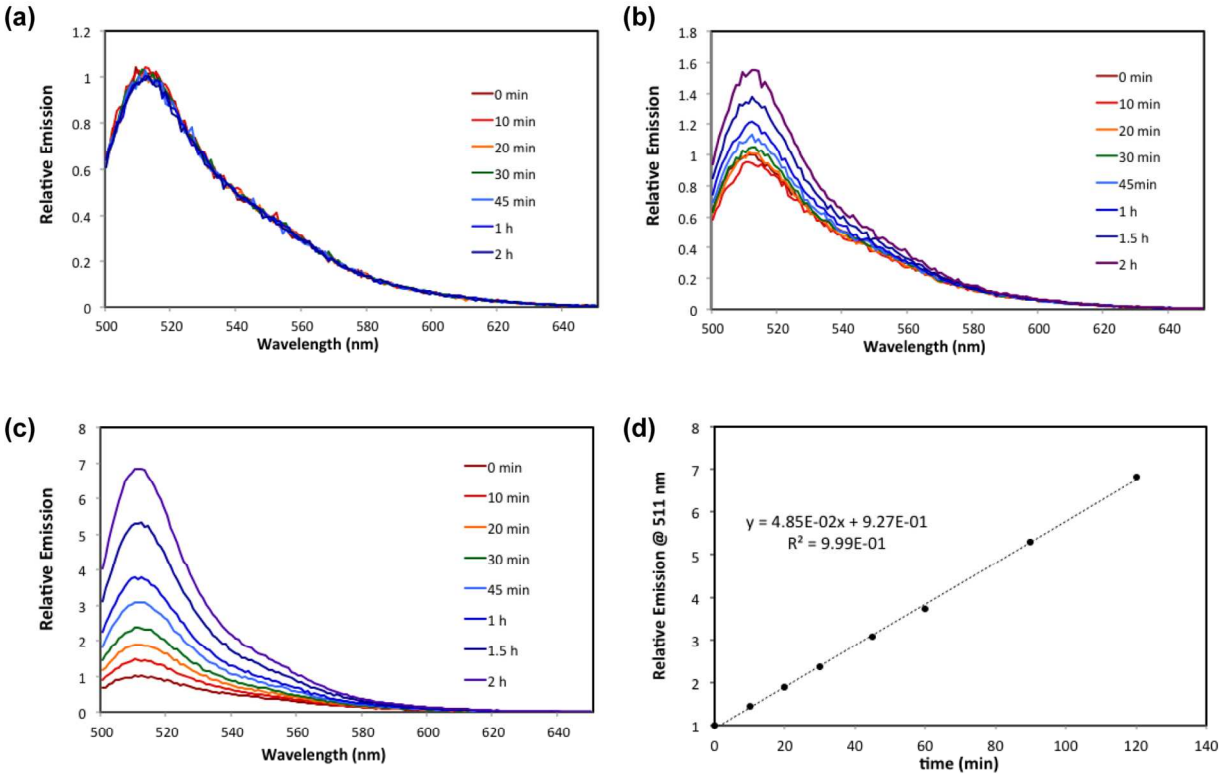
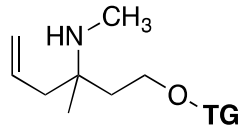


Figure S1. (a–c) Fluorescence response of **6** to varying concentrations of FA. Data was acquired in 20 mM PBS (pH 7.4) at 37 °C. Emission was collected between 500–650 nm (λ_{ex} = 488 nm). Lines represent time points taken at 0, 30, 60, 90, and 120 min after addition of (a) 0 µM, (b), 100 µM, (c) 1 mM FA. (d) Time dependent fluorescence response of **6** to 1 mM FA before saturation kinetics were observed.



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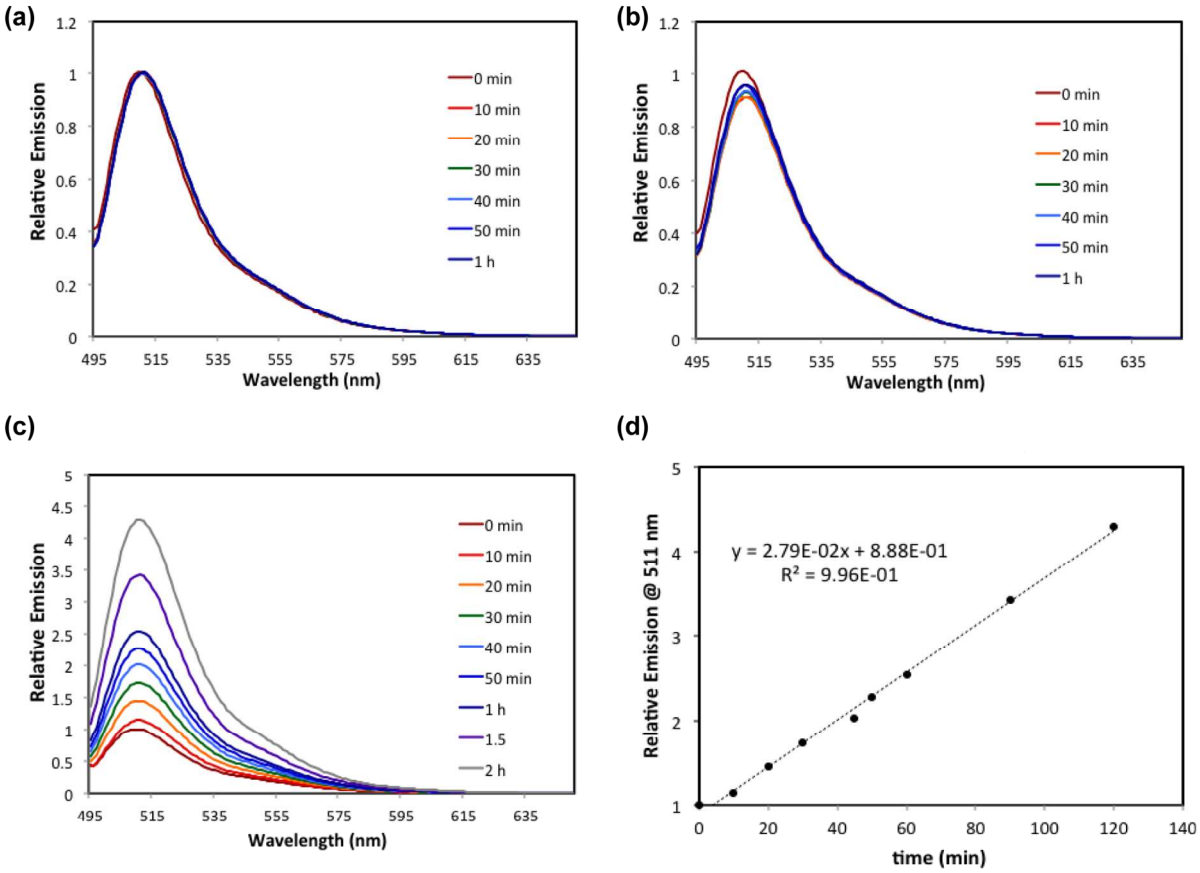
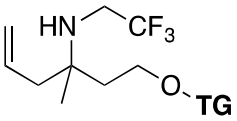


Figure S2. (a–c) Fluorescence response of **9** to varying concentrations of FA. Data was acquired in 20 mM PBS (pH 7.4) at 37 °C. Emission was collected between 495–645 nm (λ_{ex} = 488 nm). Lines represent time points taken at 0, 30, 60, 90, and 120 min after addition of (a) 0 µM, (b), 100 µM, (c) 1 mM FA. (d) Time dependent fluorescence response of **9** to 1 mM FA before saturation kinetics were observed.



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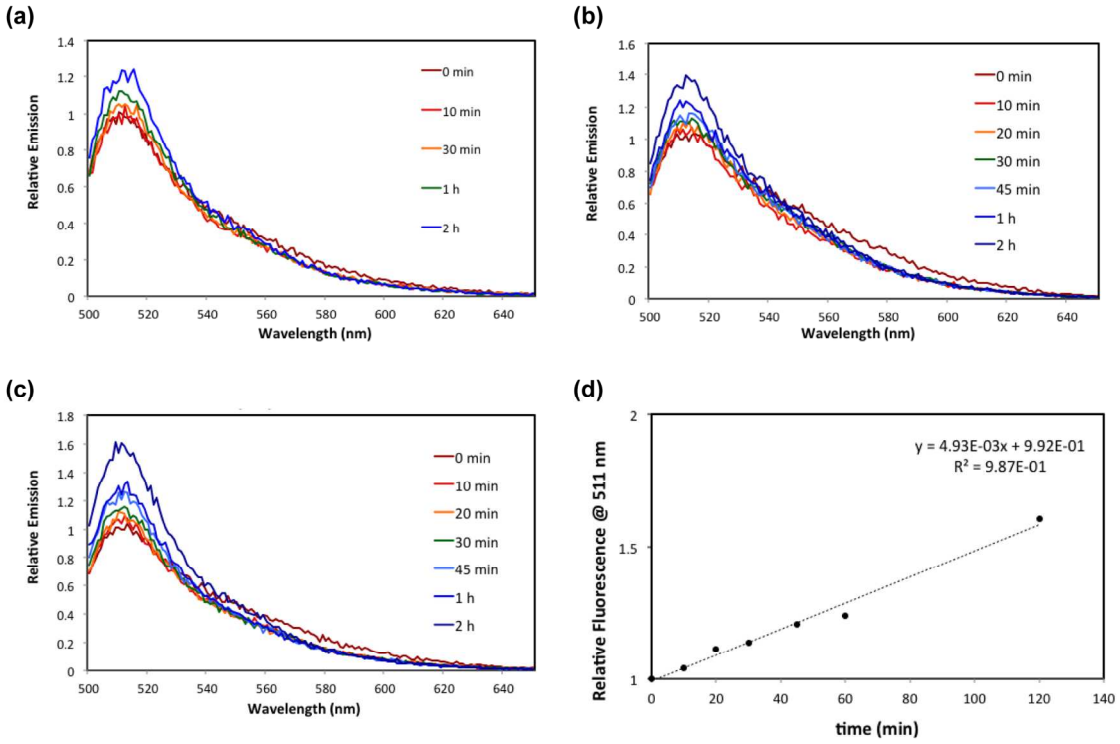


Figure S3. (a–c) Fluorescence response of **10** to varying concentrations of FA. Data was acquired in 20 mM PBS (pH 7.4) at 37 °C. Emission was collected between 500–650 nm (λ_{ex} = 488 nm). Lines represent time points taken at 0, 30, 60, 90, and 120 min after addition of (a) 0 µM, (b), 100 µM, (c) 1 mM FA. (d) Time dependent fluorescence response of **10** to 1 mM FA before saturation kinetics were observed.

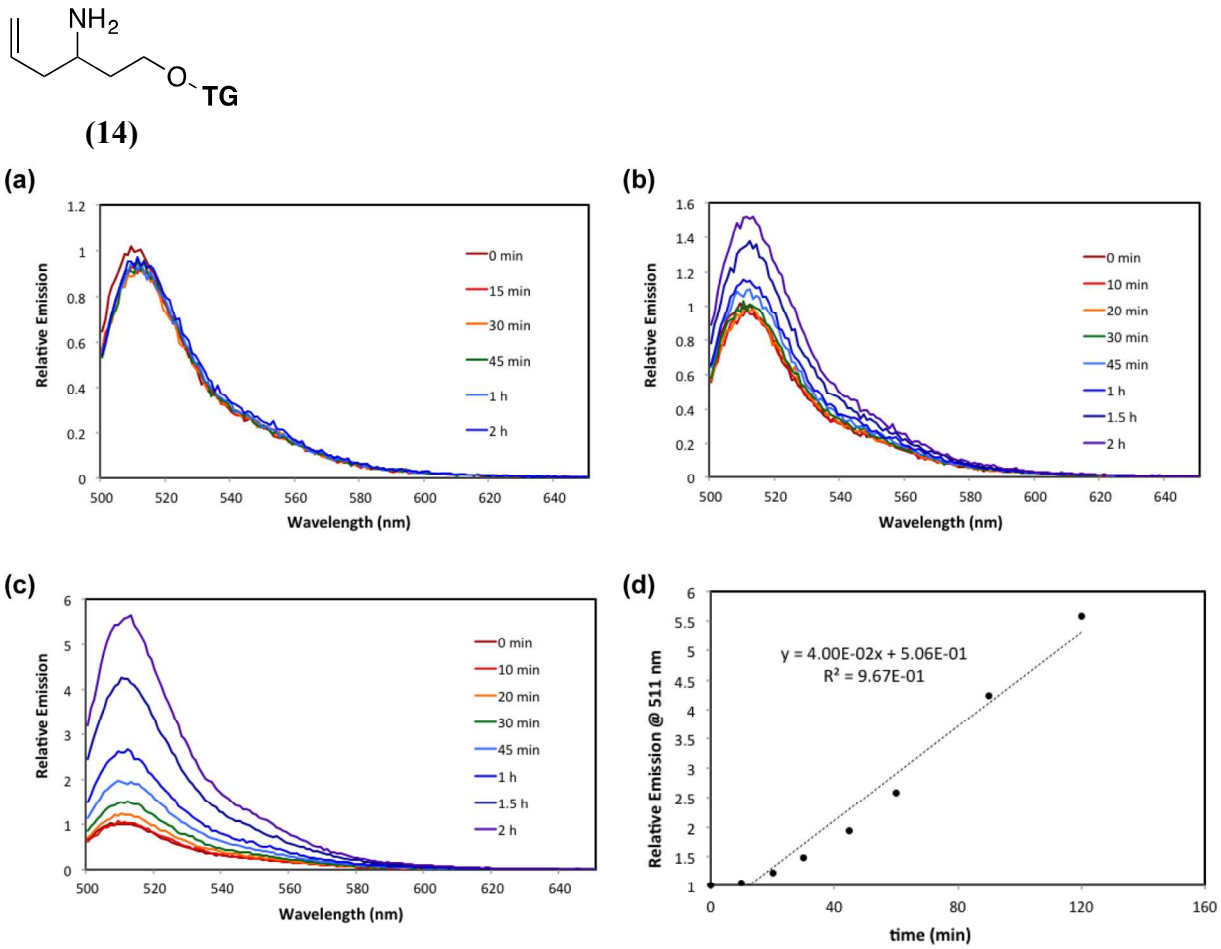
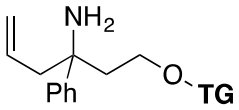


Figure S4. (a–c) Fluorescence response of **14** to varying concentrations of FA. Data was acquired in 20 mM PBS (pH 7.4) at 37 °C. Emission was collected between 500–650 nm (λ_{ex} = 488 nm). Lines represent time points taken at 0, 30, 60, 90, and 120 min after addition of (a) 0 µM, (b), 100 µM, (c) 1 mM FA. (d) Time dependent fluorescence response of **14** to 1 mM FA before saturation kinetics were observed.



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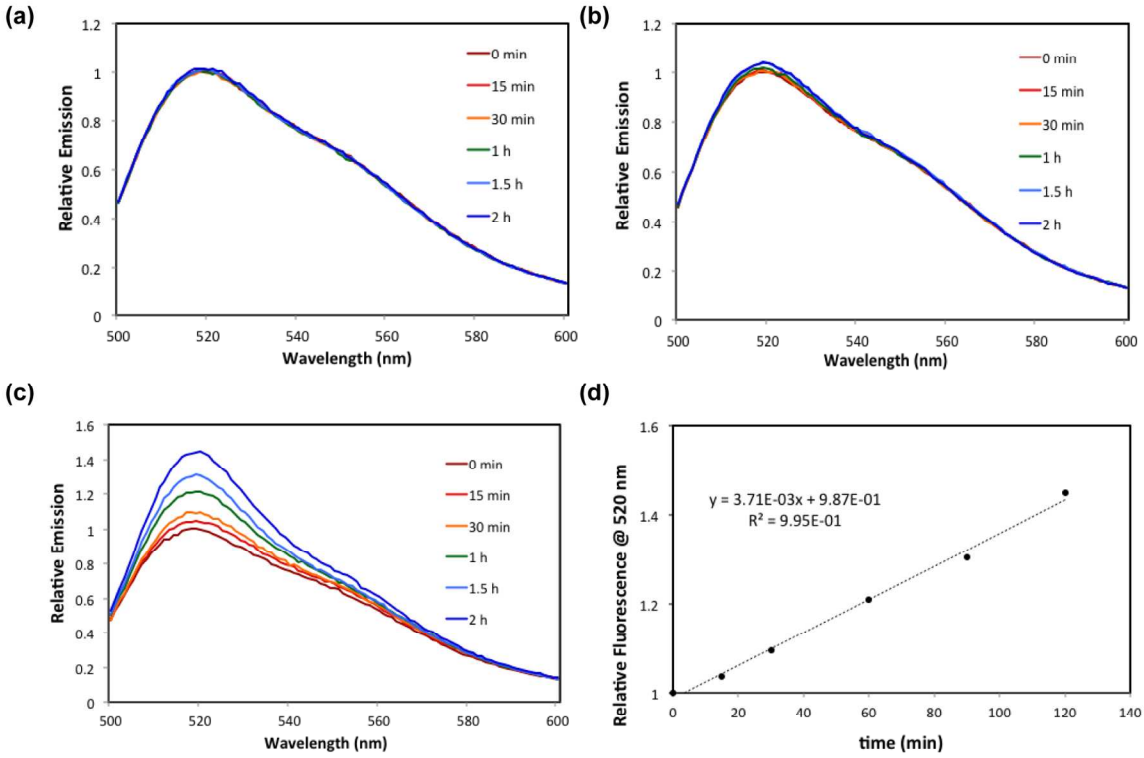
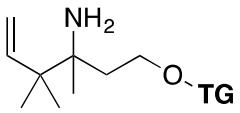


Figure S5. (a–c) Fluorescence response of **17** to varying concentrations of FA. Data was acquired in 20 mM PBS (pH 7.4) at 37 °C. Emission was collected between 500–600 nm (λ_{ex} = 488 nm). Lines represent time points taken at 0, 30, 60, 90, and 120 min after addition of (a) 0 µM, (b), 100 µM, (c) 1 mM FA. (d) Time dependent fluorescence response of **17** to 1 mM FA before saturation kinetics were observed.



(FAP488)

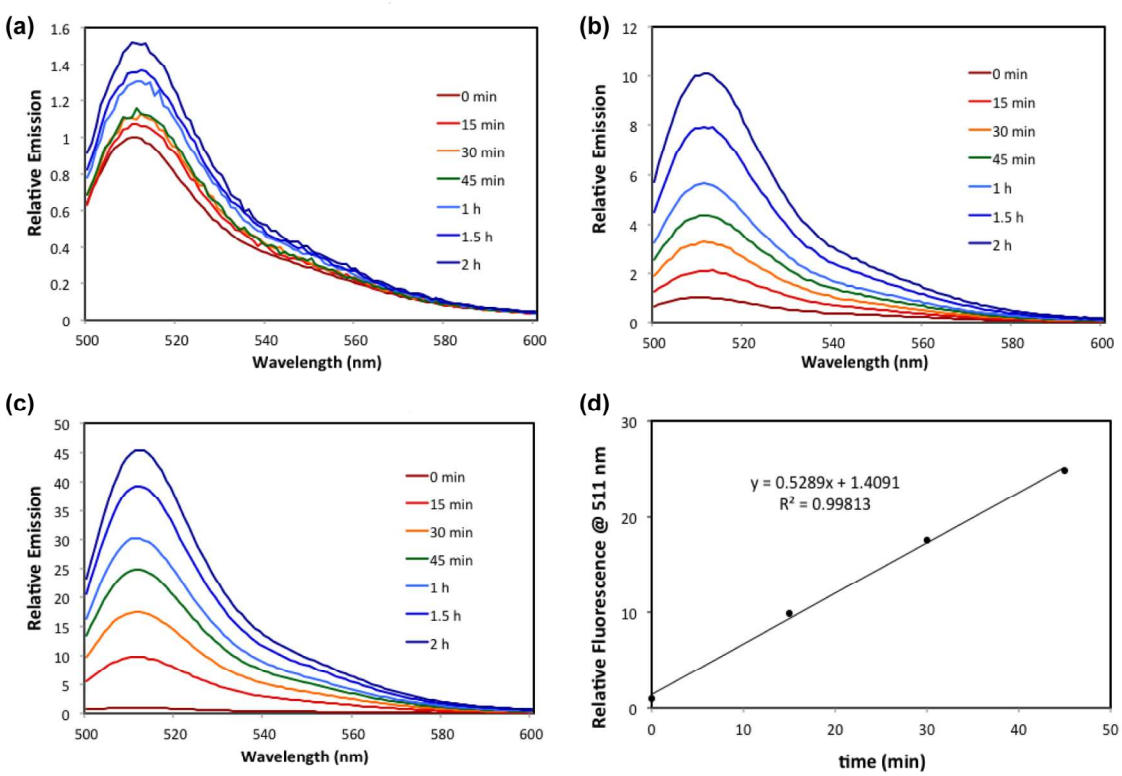


Figure S6. (a–c) Fluorescence response of **FAP488** to varying concentrations of FA. Data was acquired in 20 mM PBS (pH 7.4) at 37 °C. Emission was collected between 500–650 nm (λ_{ex} = 488 nm). Lines represent time points taken at 0, 30, 60, 90, and 120 min after addition of (a) 0 μM, (b), 100 μM, (c) 1 mM FA. (d) Time dependent fluorescence response of **FAP488** to 1 mM FA before saturation kinetics were observed.

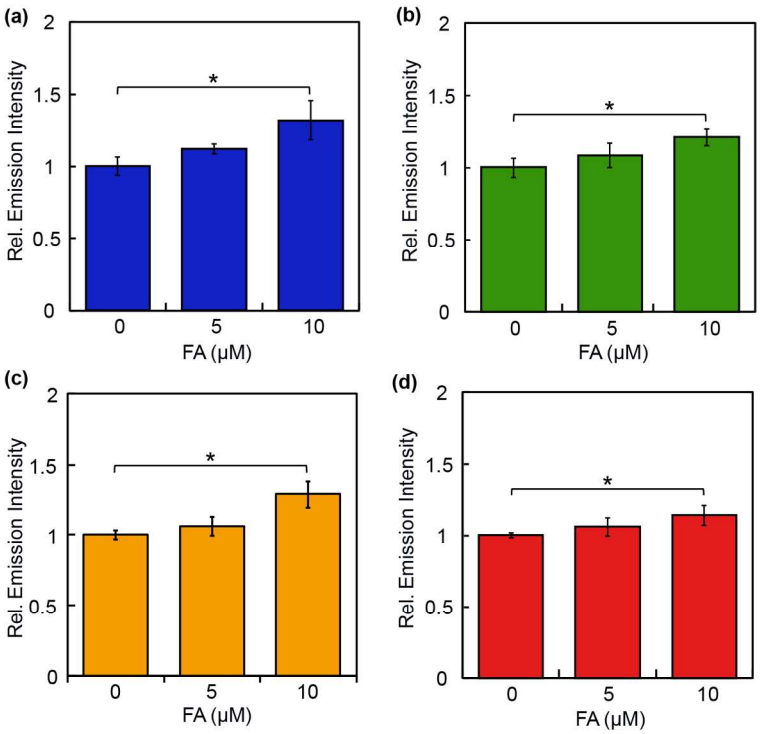


Figure S7. Probe *in vitro* Limit of Detection**.** 999 μL of a 10 μM solution of **(a) FAP385**, **(b) FAP498**, **(c) FAP555** or **(d) FAP573** in 20 mM PBS buffer (pH 7.4) was prepared by adding 1 μL of a 10 mM stock of probe in DMSO to 998 μL PBS buffer in a 1-cm x 1-cm quartz cuvette. 1 μL of a FA stock solution (either 10 mM or 5 mM, prepared on the same day by diluting commercial 37 wt % FA solution in Milli-Q water) or 1 μL PBS was added (final concentration 10, 5, or 0 μM FA), and mixed by vigorous pipetting. The cuvette was placed in a 37 °C water bath, and emission spectra were collected after 120 minutes. Statistical analysis of three replicates using the software, R, was performed using a one-way ANOVA with a Bonferroni correction. * $P < 5E{-}3$.

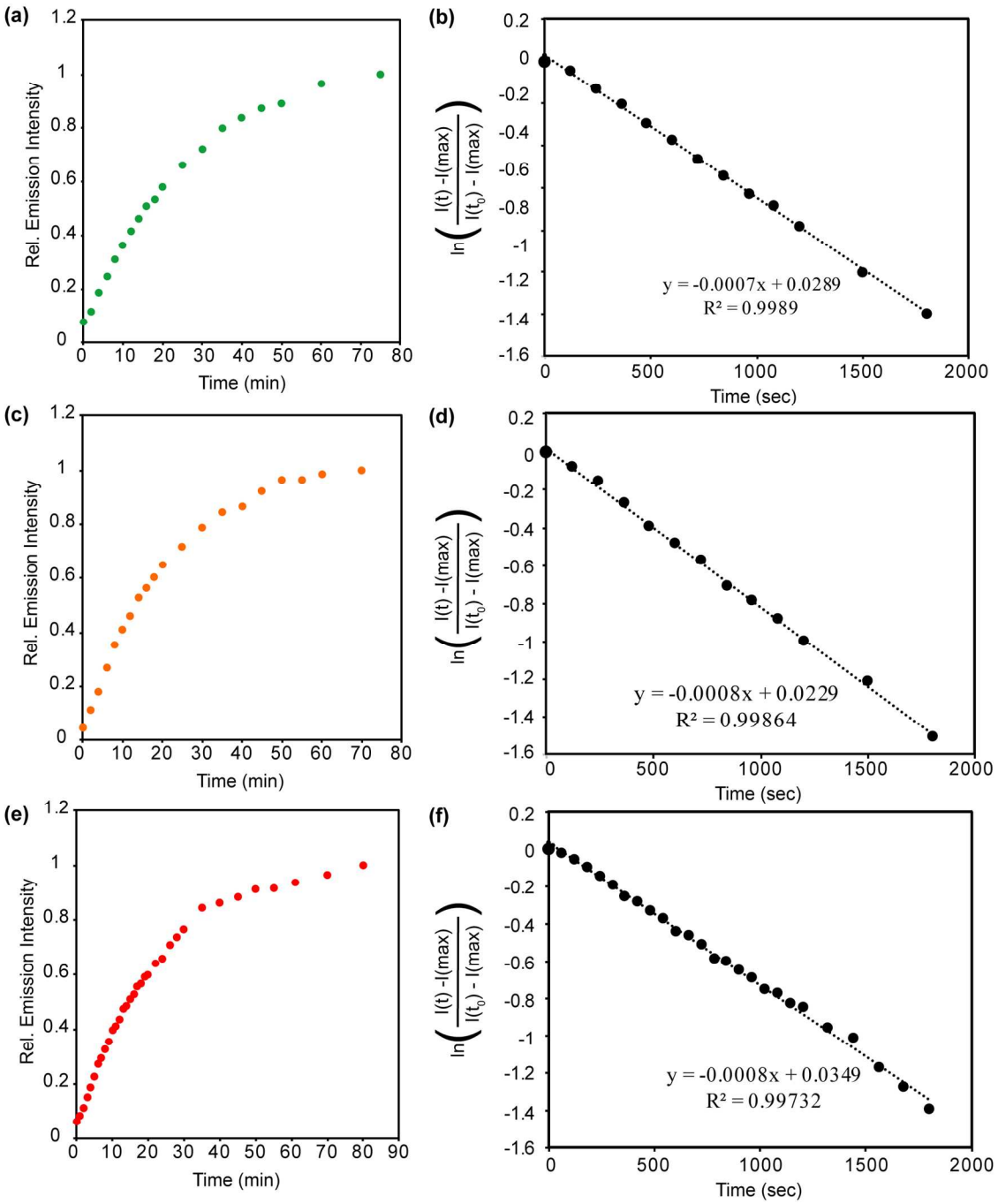


Figure S8. **(a)** Kinetic profile of 10 μM **FAP488** reacting with 5 mM FA monitored at 515 nm. The reaction was carried out at 37 °C in PBS buffer (pH 7.4). **(b)** Linearized integrated rate law assuming pseudo first order kinetics. Bimolecular rate constant (linearized integrated rate law/[FA]): k_{obs}= 0.14 ± 0.01 M^{-1} s^{-1}. **(c)** Kinetic profile of 10 μM **FAP555** reacting with 5 mM FA monitored at 572 nm. The reaction was carried out at 37 °C in PBS buffer (pH 7.4). **(d)** Linearized integrated rate law assuming pseudo first order kinetics. Bimolecular rate constant (linearized integrated rate law/[FA]): k_{obs}= 0.16 ± 0.01 M^{-1} s^{-1}. **(e)** Kinetic profile of 10 μM **FAP573** reacting with 5 mM FA monitored at 585 nm. The reaction was carried out at 37 °C in PBS buffer (pH 7.4). **(f)** Linearized integrated rate law assuming pseudo first order kinetics. Bimolecular rate constant (linearized integrated rate law/[FA]): k_{obs}= 0.16 ± 0.02 M^{-1} s^{-1}.

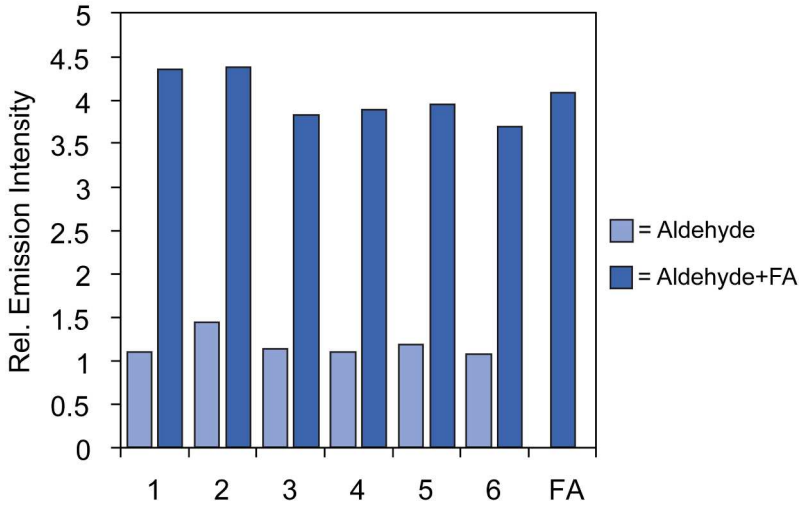


Figure S9. Aldehyde fluorescence turn-on competition experiment**.** 980 µL of a 10 µM solution of **FAP573** in 20 mM PBS buffer (pH 7.4) was prepared by adding 1 µL of a 10 mM stock of probe in DMSO to 979 µL PBS buffer in a 1-cm x 1-cm quartz cuvette. 10 µL of an aldehyde stock solution (final concentration 100 µM) was added, followed by 10 µL of a FA stock solution (final concentration 100 µM) and mixed by vigorous pipetting. The cuvette was placed in a 37 °C water bath, and emission spectra were collected after 120 minutes. Legend: (1) acrolein, (2) methylglyoxal, (3) acetaldehyde, (4) 4-hydroxynonenal, (5) glucosone, (6) oxaloacetate.

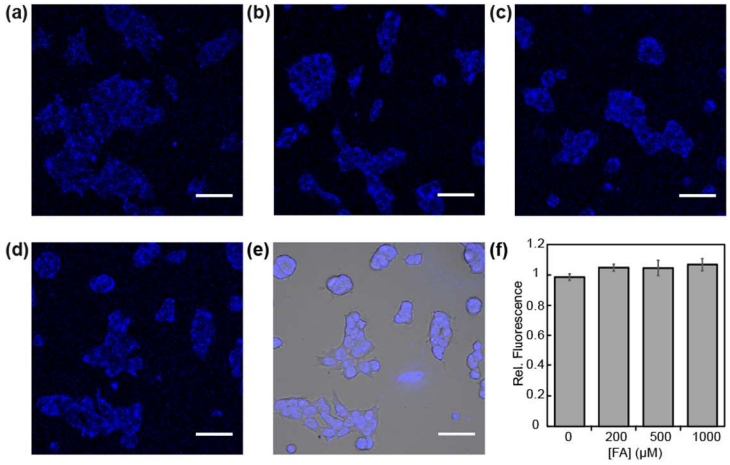


Figure S10. Confocal microscopy images of **FAP385** in response to exogenous FA addition in HEK293T cells. Cells were treated with 10 µM **FAP385** in BSS buffer for 30 minutes, exchanged into fresh buffer, and then treated with (a) vehicle, (b) 200, (c) 500, or (d) 1000 µM FA. Images were taken after 60 min. (e) Bright field image of cells in (d) overlaid with 1 µM Hoechst 33342. (f) Mean fluorescent intensities of cells in (a)-(d) 60 min after addition of FA relative to mean fluorescence intensity before addition of vehicle or FA; error bars denote SEM (n=3).

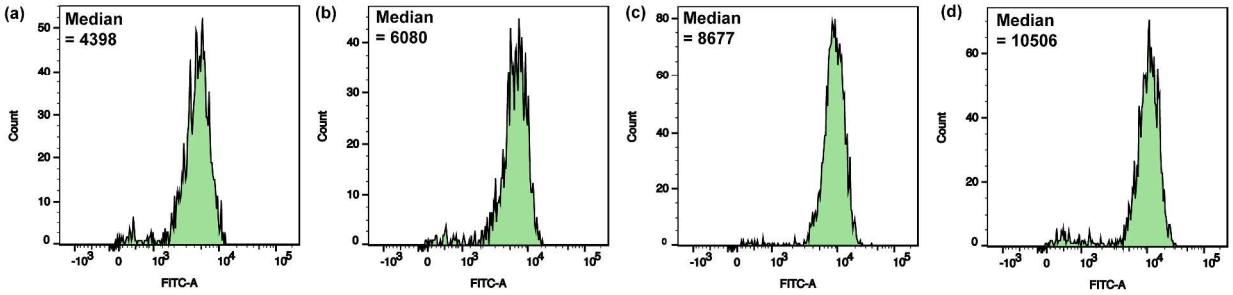


Figure S11. Representative experiment for flow cytometry of **FAP498** in response to exogenous FA (for summary of three technical replicates see Figure S19). HEK293T cells were plated in a 12-well polystyrene culture plate (Corning) and allowed to grow to the same confluence as in the corresponding confocal experiment. The cells were incubated with **(a)** 0, **(b)** 200, **(c)** 500, or **(d)** 1000 μM FA in BSS buffer at 37 ºC for 60 min.

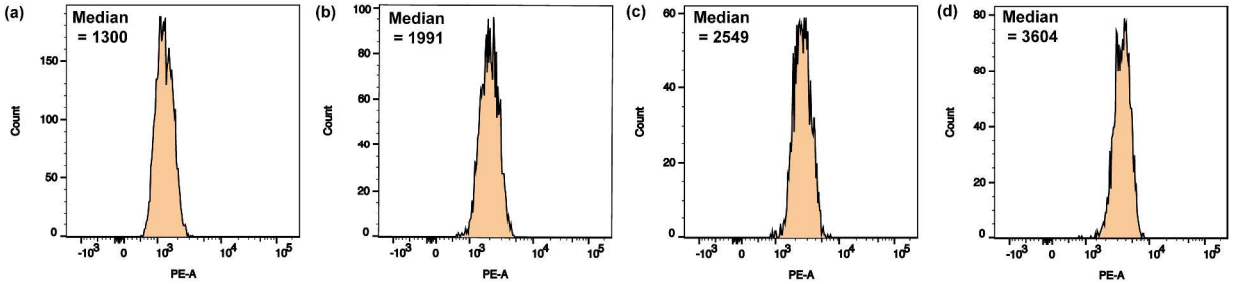


Figure S12. Representative experiment for flow cytometry of **FAP555** in response to exogenous FA (for summary of three technical replicates see Figure S19). HEK293T cells were plated in a 12-well polystyrene culture plate (Corning) and allowed to grow to the same confluence as in the corresponding confocal experiment. The cells were incubated with **(a)** 0, **(b)** 200, **(c)** 500, or **(d)** 1000 μM FA in BSS buffer at 37 ºC for 60 min.

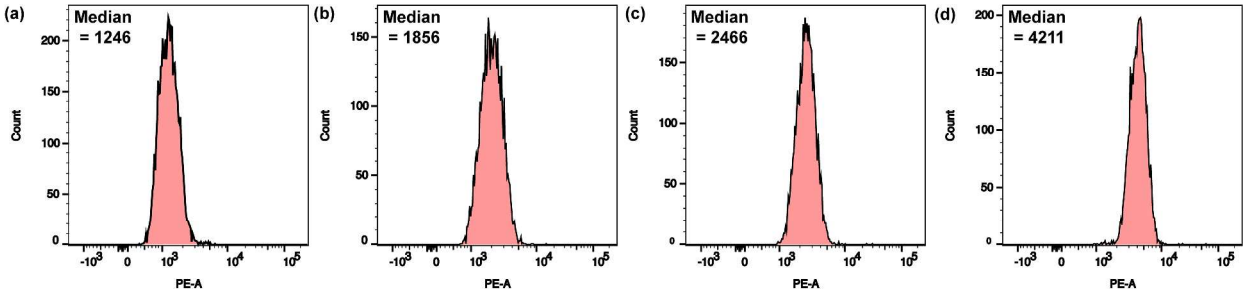


Figure S13. Representative experiment for flow cytometry of **FAP573** in response to exogenous FA (for summary of three technical replicates see Figure S19). HEK293T cells were plated in a 12-well polystyrene culture plate (Corning) and allowed to grow to the same confluence as in the corresponding confocal experiment. The cells were incubated with **(a)** 0, **(b)** 200, **(c)** 500, or **(d)** 1000 μM FA in BSS buffer at 37 ºC for 60 min.

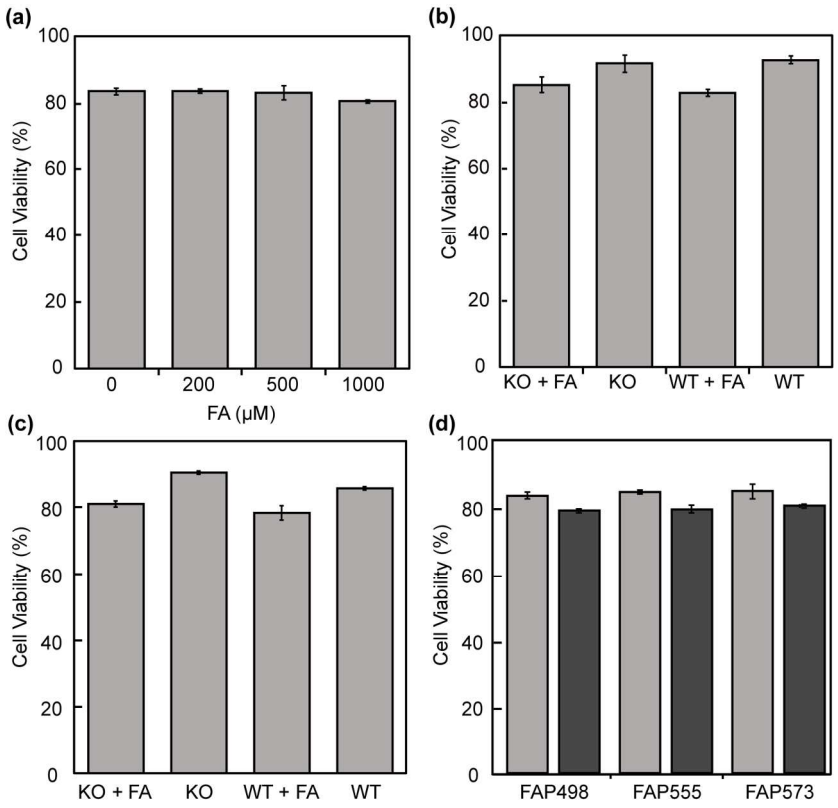


Figure S14. Cell viability experiment using flow cytometry with propidium iodide (PI) (for FAP488) or Sytox Red (for FAP555 and FAP573) staining. Cells were plated in a 6-well polystyrene culture plate (Corning) and allowed to grow to the same confluence as in the corresponding confocal experiment. The cells were treated as stated below, exchanged into 3 μM PI or 5 μM Sytox Red in PBS buffer at 37 °C, incubated for 5 min, and then gently dislodged and filtered into a 12x75 mm polysterene tube (Corning) fitted with 35 μm nylon mesh cap. **(a)** HEK293T cells were incubated with 0, 200, 500, or 1000 μM FA in BSS buffer at 37 ºC for 60 min, **(b)** MEF ADH3 KO cells and MEF WT cells were incubated with 100 or 0 μM FA in BSS buffer at 37 °C for 60 min, **(c)** HAP1 ADH3 KO cells and HAP1 WT cells were incubated with 100 or 0 μM FA in BSS buffer at 37 °C for 60 min, and **(d)** HEK293T cells were incubated with 10 μM probe (light gray) or 10 μM probe and 1 mM FA (dark gray) at 37 °C for 60 min.

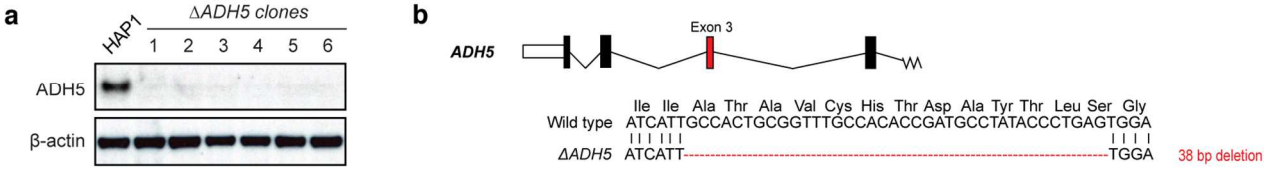


Figure S15. Disruption of ADH5 in HAP1. **(a)** Western blot analysis of *ADH5* knockout clones in HAP1 cells. **(b)** Targeted exon (red) and genetic changes of HAP1 *ADH5* knockout clone.

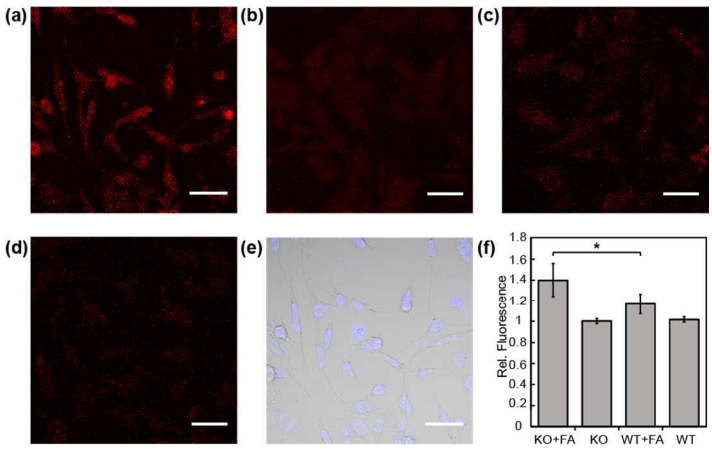


Figure S16. Confocal microscopy images of FAP573 in response to FA metabolism in ADH5 KO (a,b) or WT (c,d) MEF cells. Cells were treated with 10 μM FAP573 for 30 min, exchanged into fresh buffer, and then treated with vehicle (b,d) or 100 μM FA (a,c). Images were taken after 60 min. (e) Bright field image of (a) overlaid with 1 μM Hoechst 33342. (f) Mean fluorescence intensities of cells in (a)-(d) 60 min after addition of FA relative to mean fluorescence intensity before addition of vehicle or FA; error bars denote SEM (n=3). * P < 5E–3.

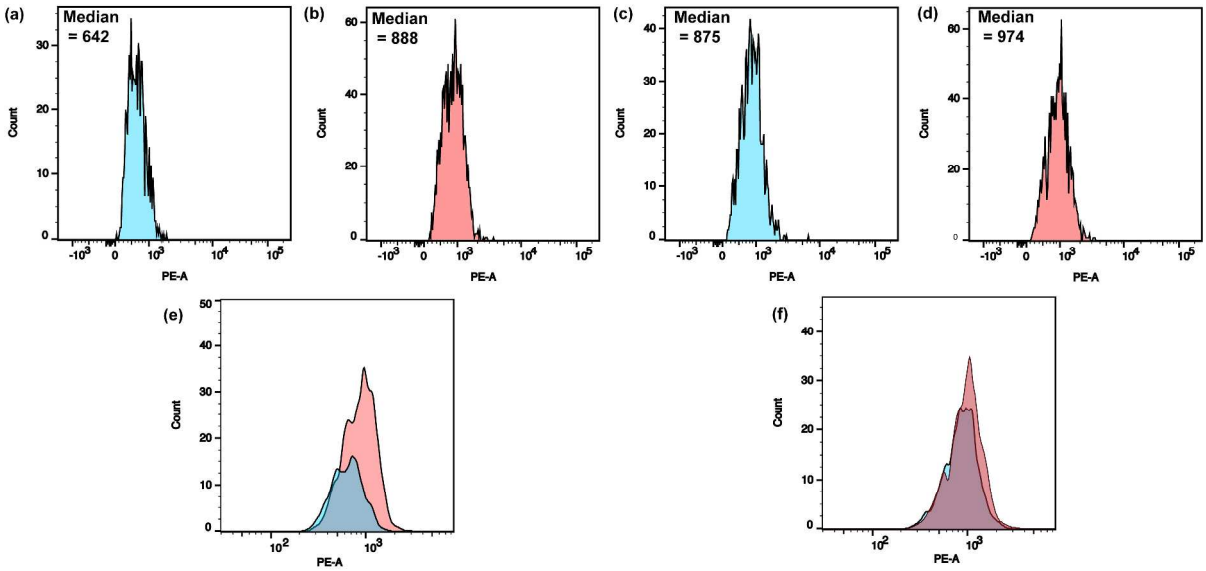


Figure S17. Representative experiment for flow cytometry of **FAP573** in response to exogenous FA in an ADH5 KO model (for summary of three technical replicates see Figure S19). HAP1 WT or ADH5 KO cells were plated in a 12-well polystyrene culture plate (Corning) and allowed to grow to the same confluence as in the corresponding confocal experiment. The KO cells were then incubated with **(a)** 0 μM or **(b)** 100 μM FA, in BSS buffer at 37 ºC for 60 min and the WT cells were incubated with **(c)** 0 μM or **(d)** 100 μM FA, in BSS buffer at 37 ºC for 60 min. **(e)** Smooth data overlay of data in (a) and (b). **(f)** Smooth data overlay of data in (c) and (d).

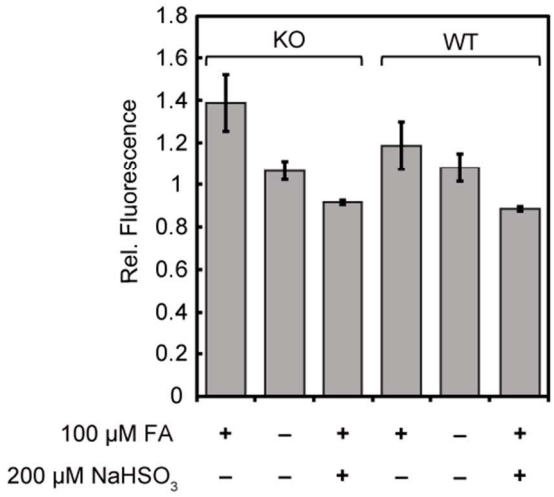


Figure S18. ADH5 KO experiment with $NaHSO_3$ addition. Cells were treated with 200 μM $NaHSO_3$ for 30 minutes, incubated with 10 μM FAP573 in buffer containing 200 μM $NaHSO_3$ for 30 min, exchanged into fresh buffer with 200 μM $NaHSO_3$, and treated with 100 μM FA. The mean fluorescent intensities of cells 60 min after addition of FA relative to mean fluorescence intensity before addition of vehicle or FA is shown.

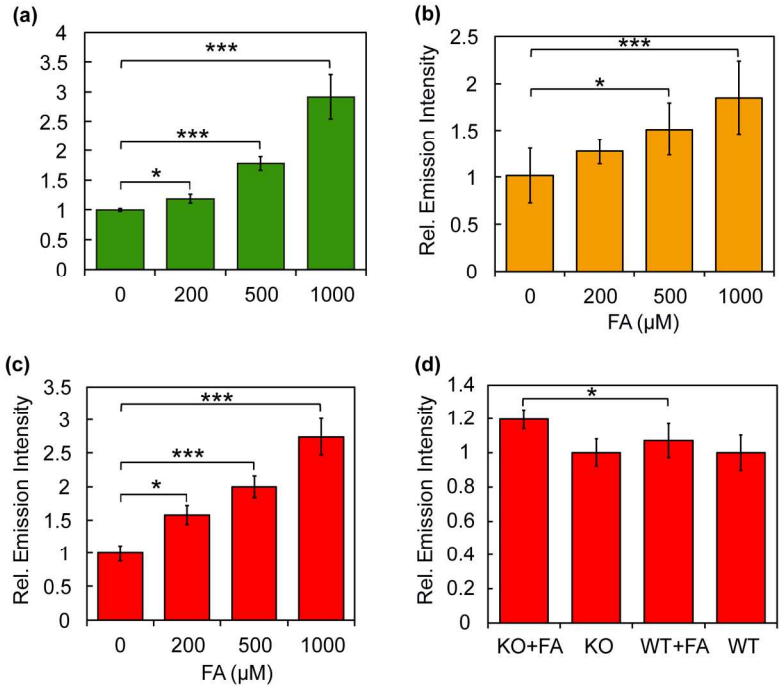
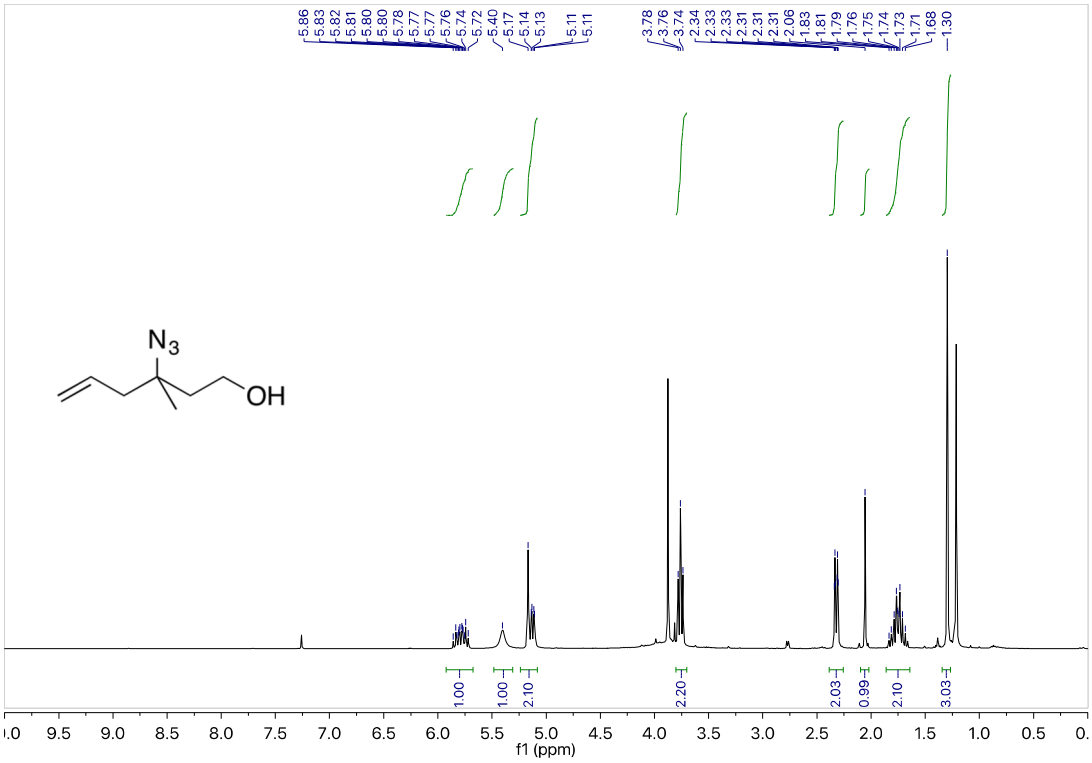
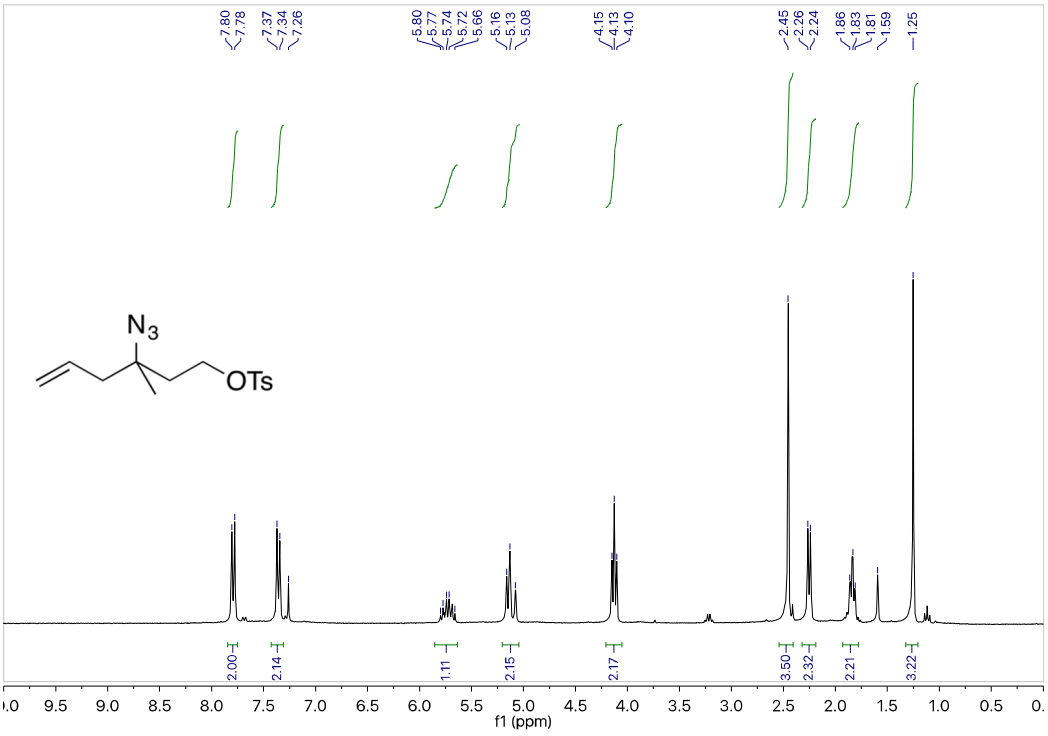
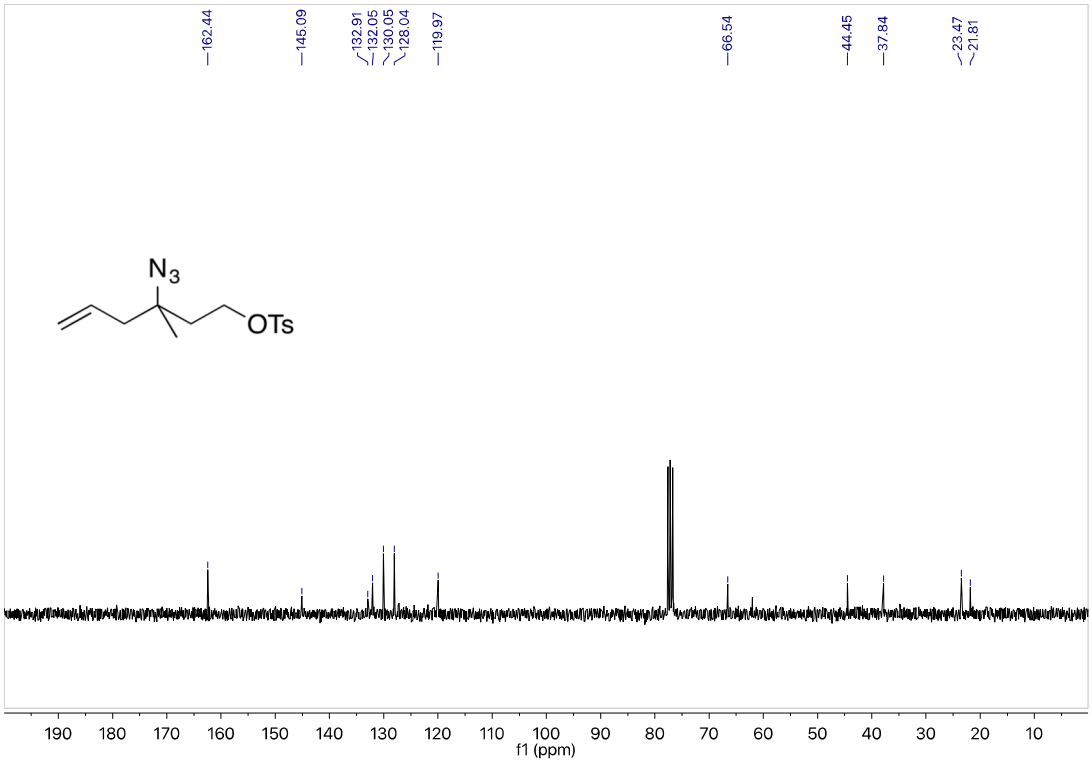


Figure S19. Summary of flow cytometry turn-on experiments with technical replicates (n=3). **(a)** Response of **FAP488** to varying concentrations of FA in HEK293T cells, **(b)** response of **FAP555** to varying concentrations of FA in HEK293T cells, **(c)** response of **FAP573** to varying concentrations of FA in HEK293T cells, and **(d)** response of **FAP573** to 100 μM FA or 0 μM FA in WT or ADH5 KO HAP1 cells. Statistical analysis of three replicates using the software, R, was performed using a one-way ANOVA with a Bonferroni correction. * P ≤ 5E–3, *** P < 5E–5.

Copies of Spectra



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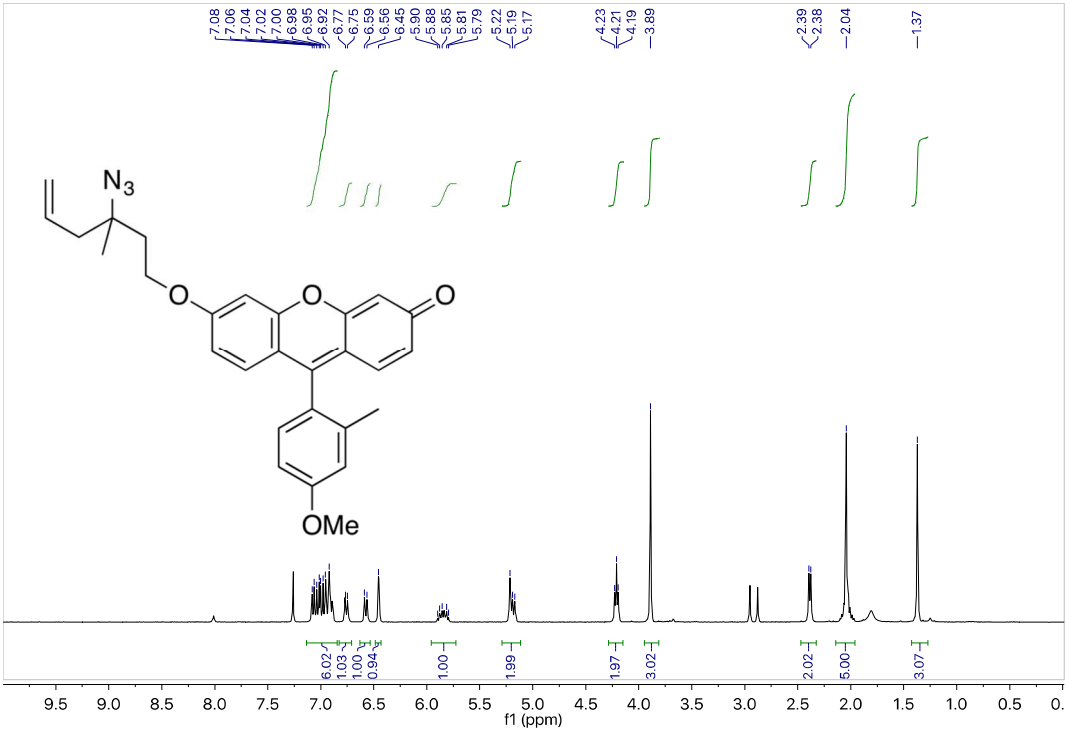
N3
OTs
f1 (ppm)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5.79
5.22
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4.19
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2.39
2.38
2.04
1.37
N3
O
O
O
OMe
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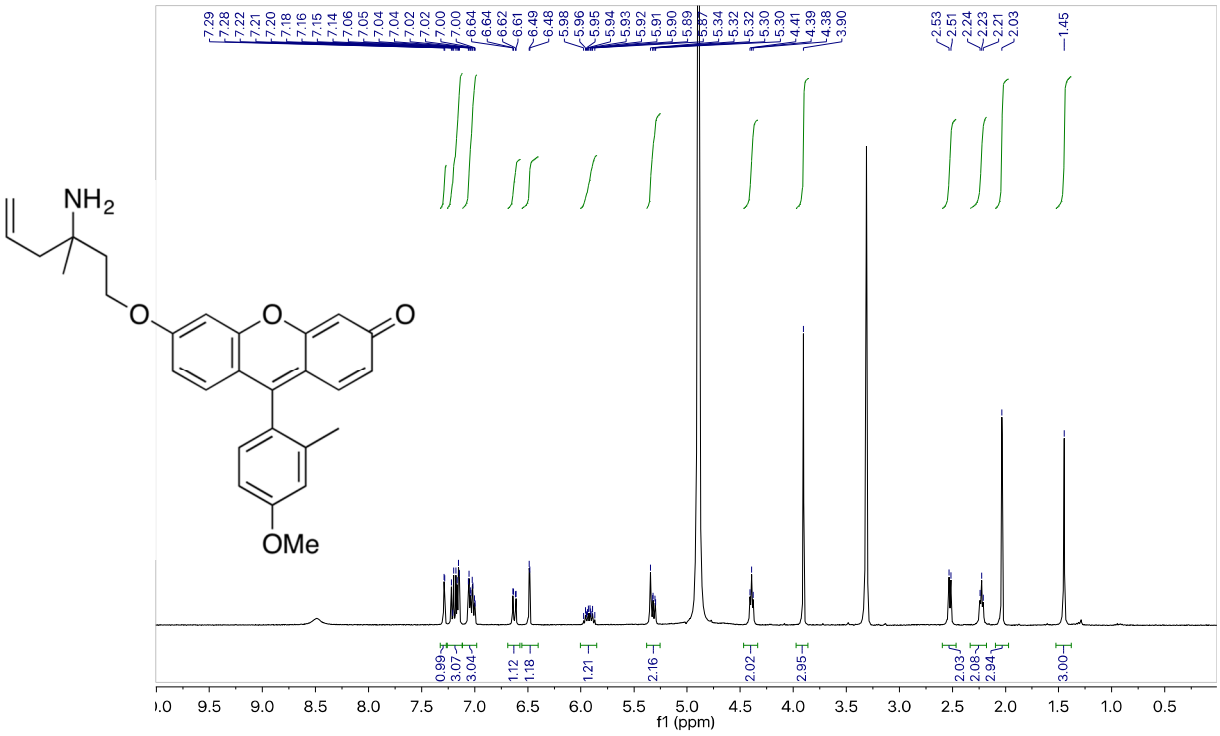
3.07
f1 (ppm)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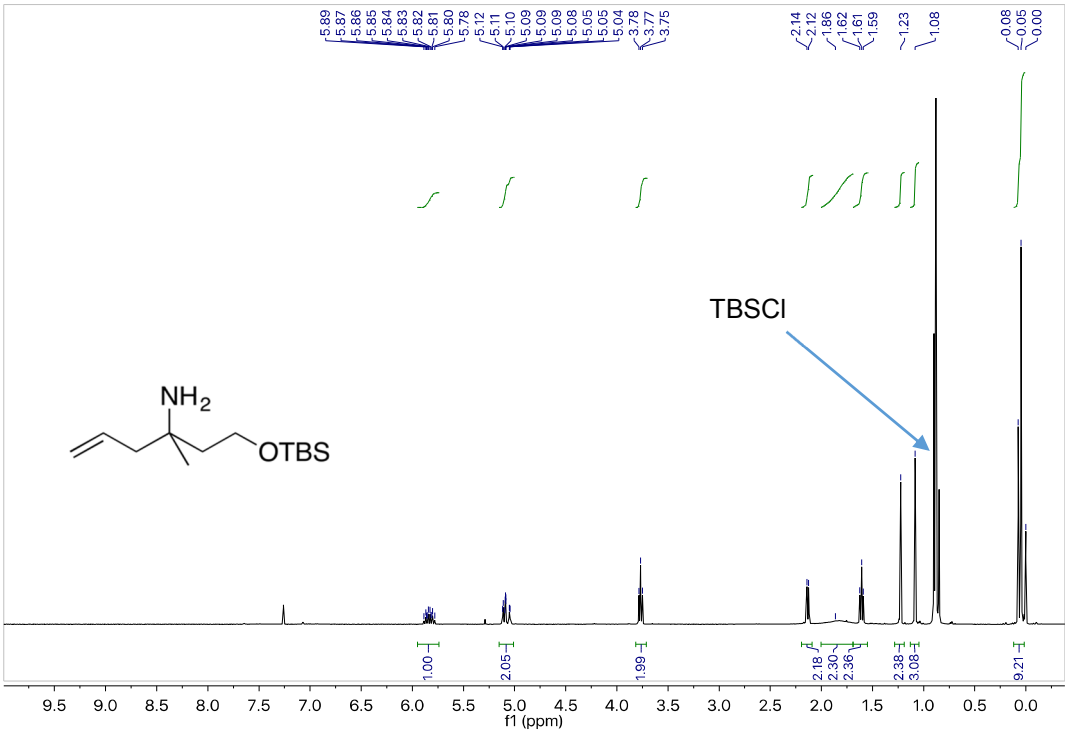
OMe
f1 (ppm)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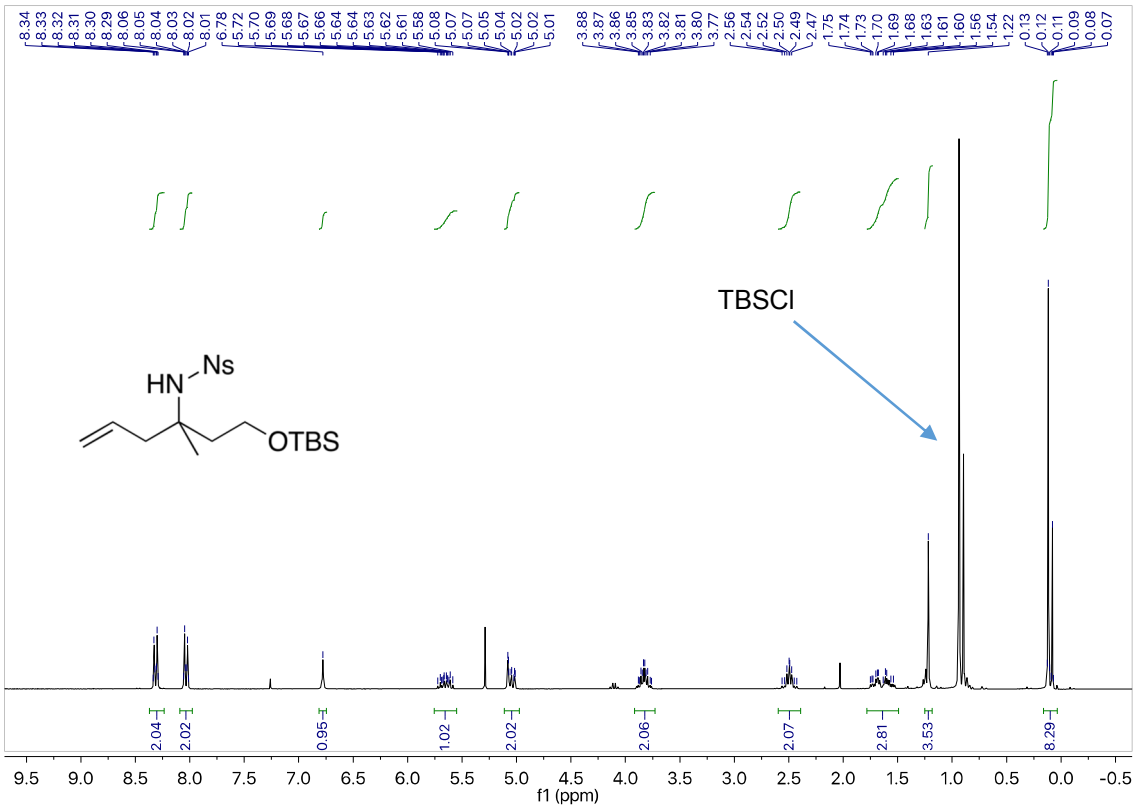
NH2
OTBS
f1 (ppm)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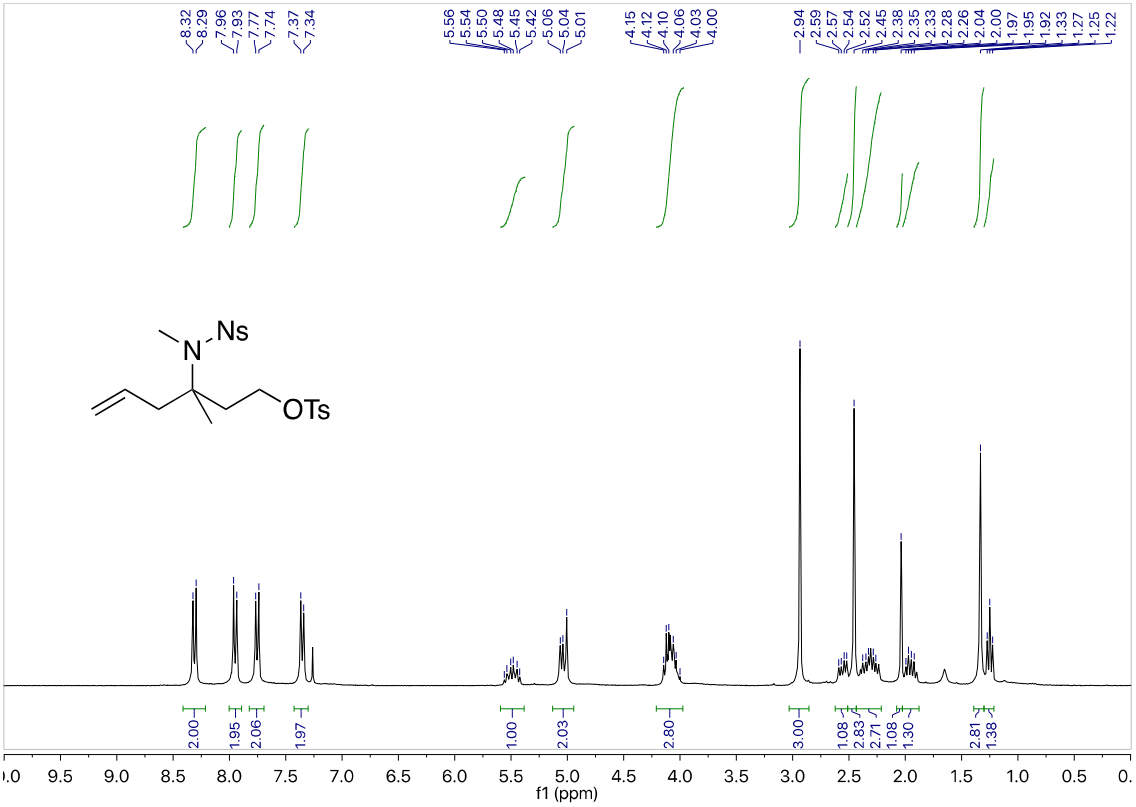
HN
Ns
OTBS
f1 (ppm)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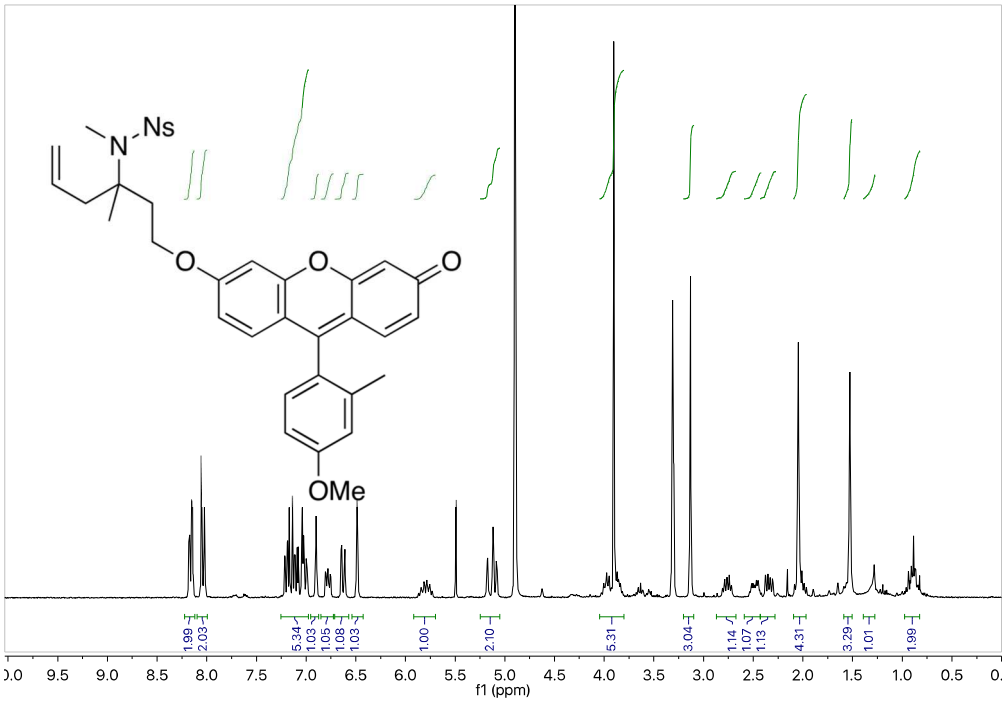
OTs
f1 (ppm)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N
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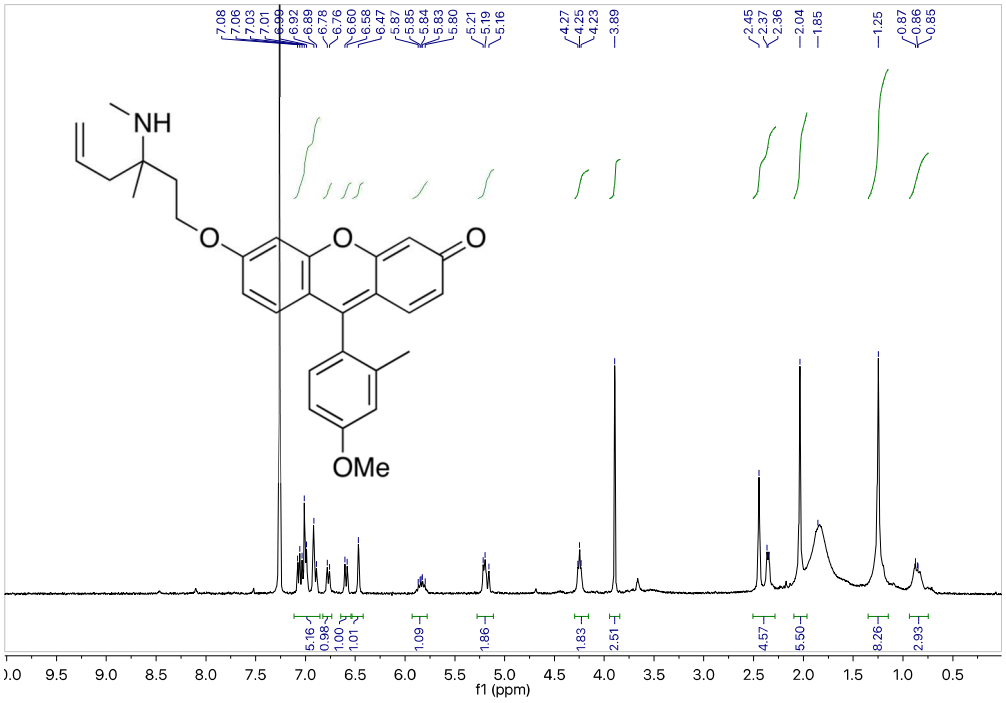
O
O
OMe
f1 (ppm)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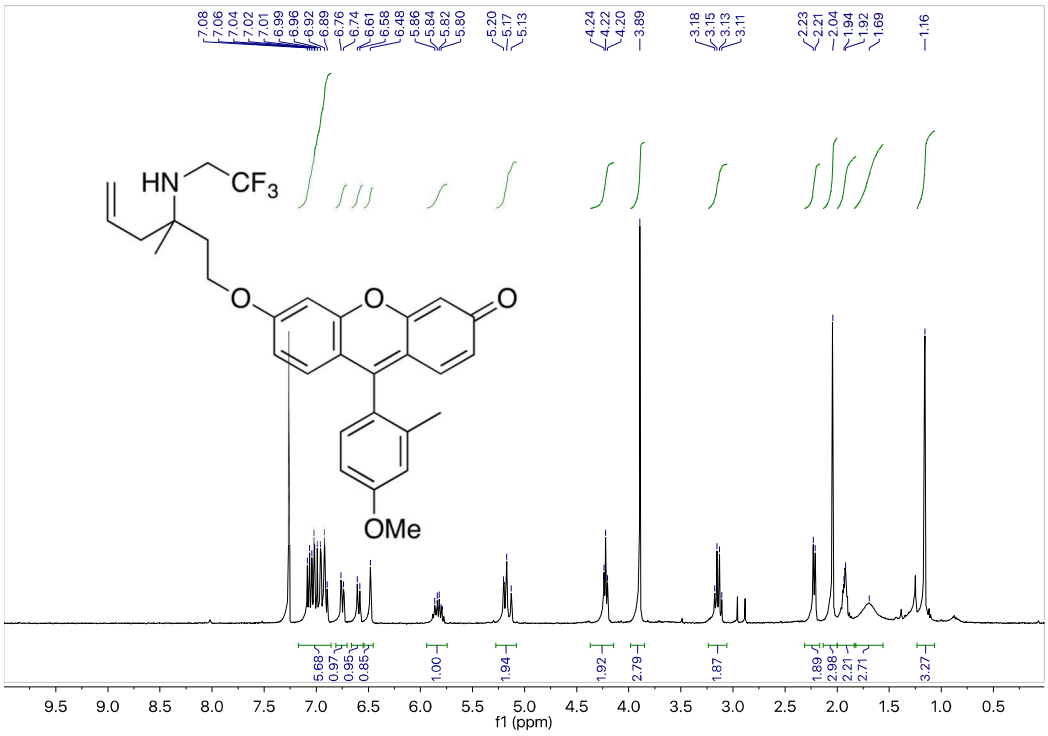
f1 (ppm)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CF3
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O
O
OMe
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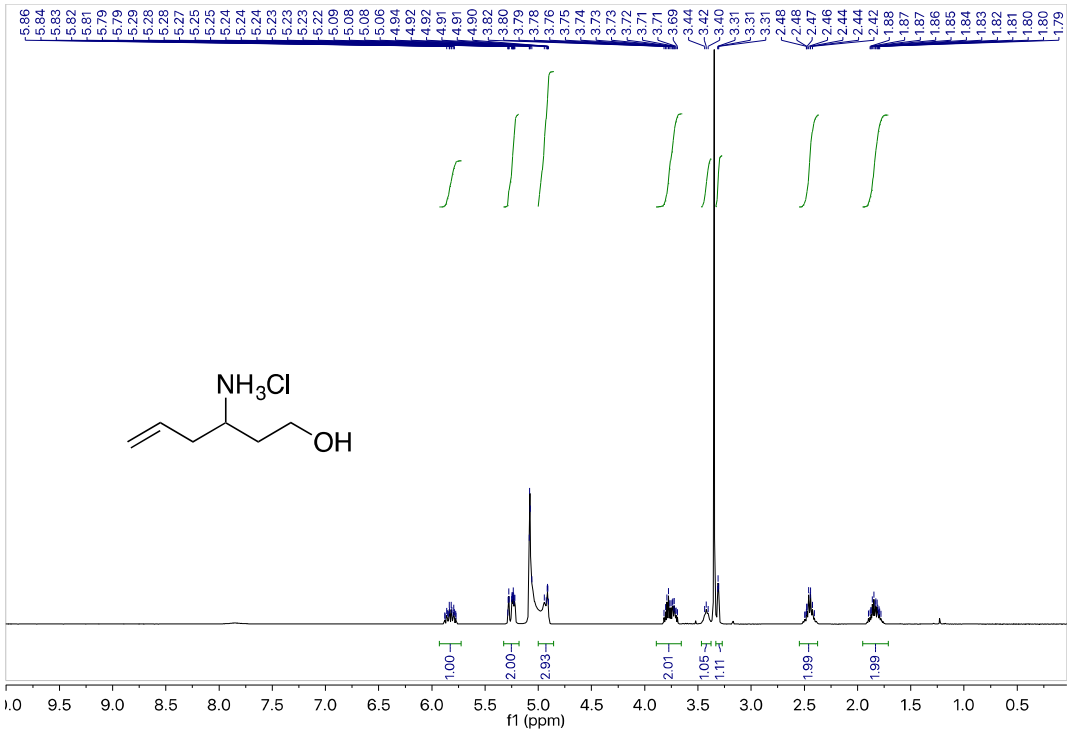
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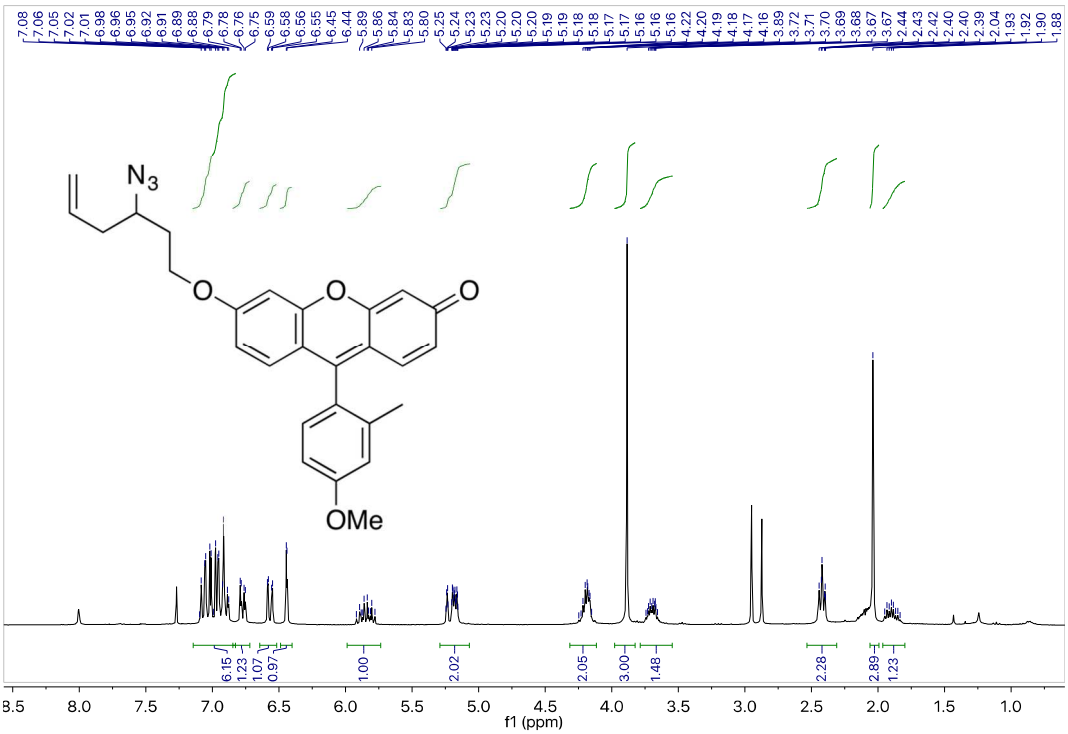
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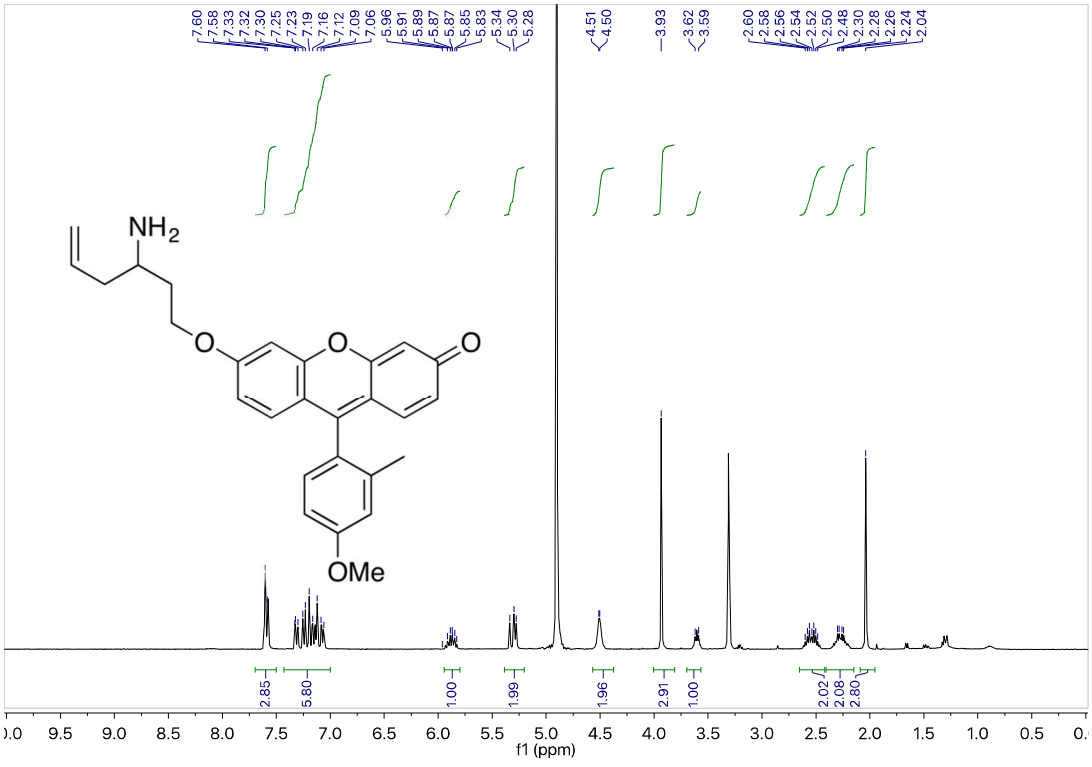
O
O
OMe
f1 (ppm)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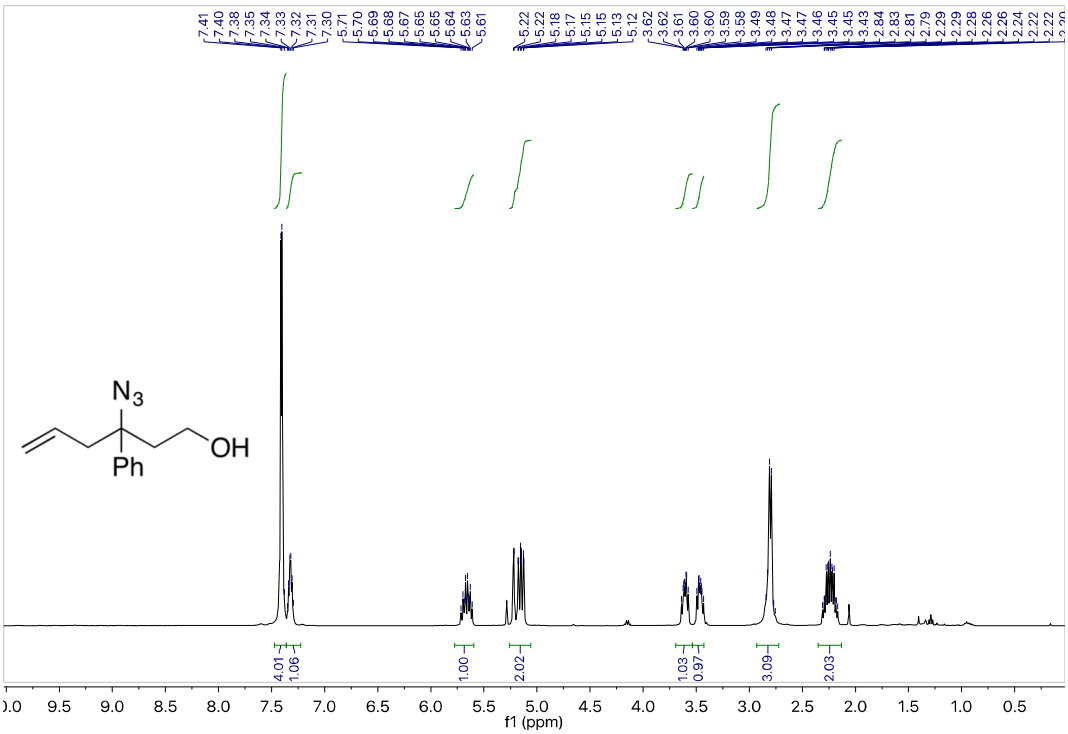
OMe
f1 (ppm)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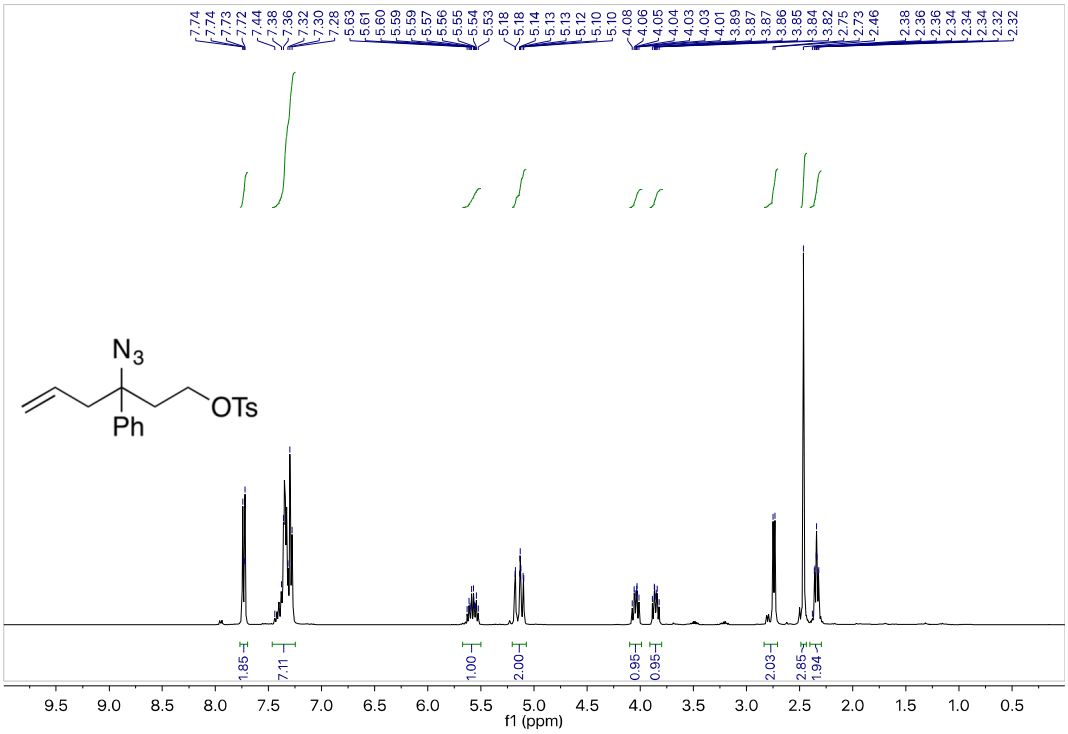
Ph
OH
f1 (ppm)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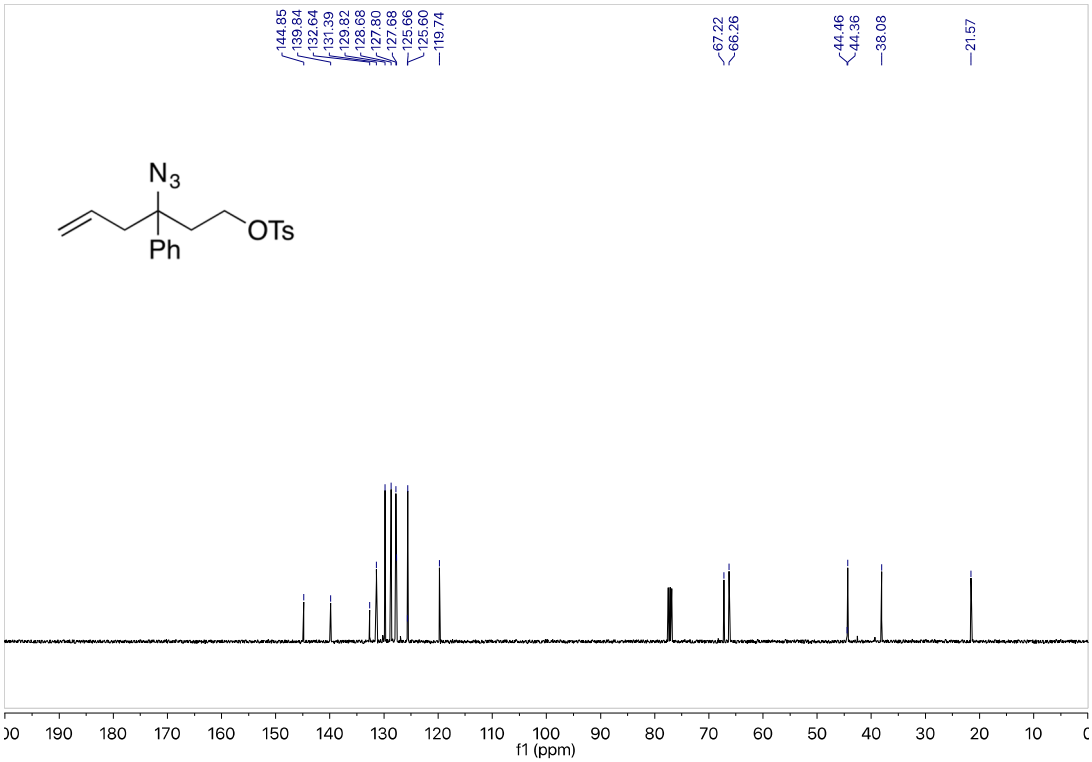
Ph
OTs
f1 (ppm)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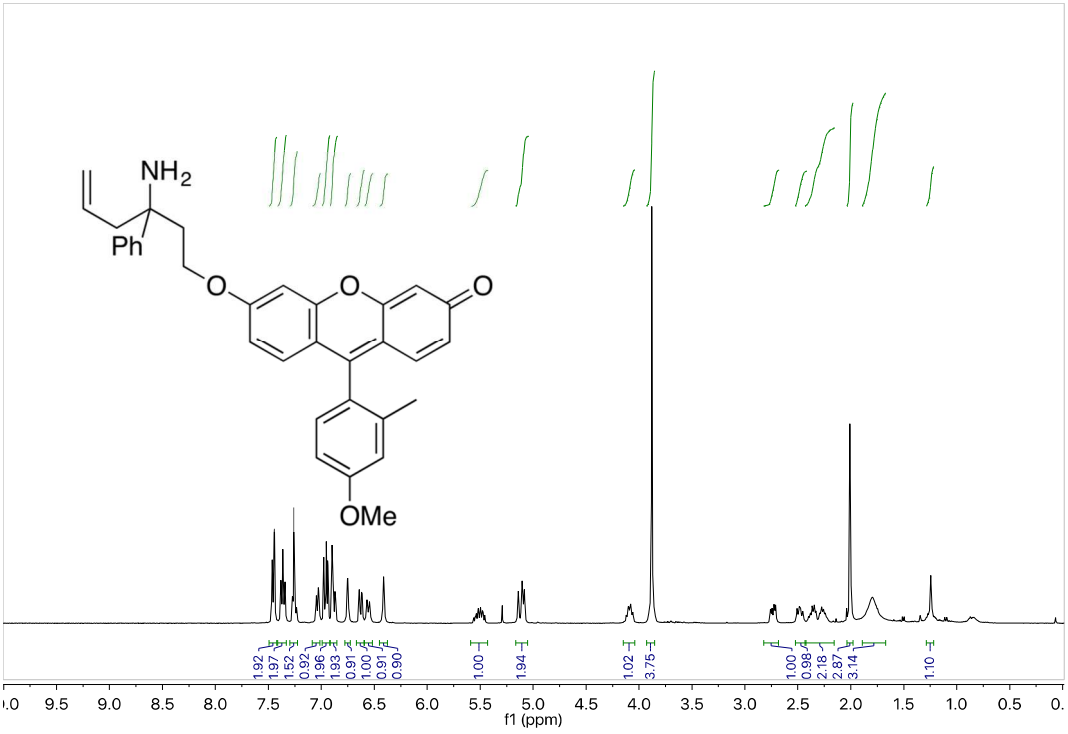
Ph
OTs
f1 (ppm)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Ph
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O
O
OMe
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1.93
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0.91
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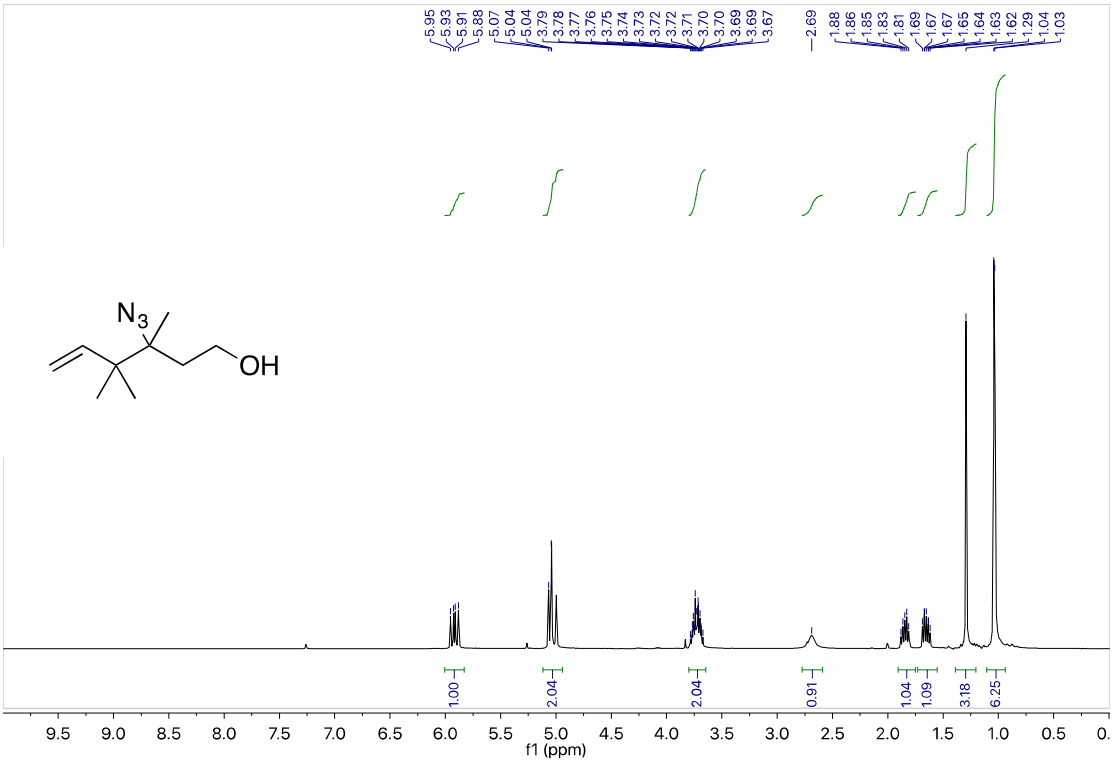
f1 (ppm)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1.67
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1.62
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1.03
N3
OH
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2.04
2.04
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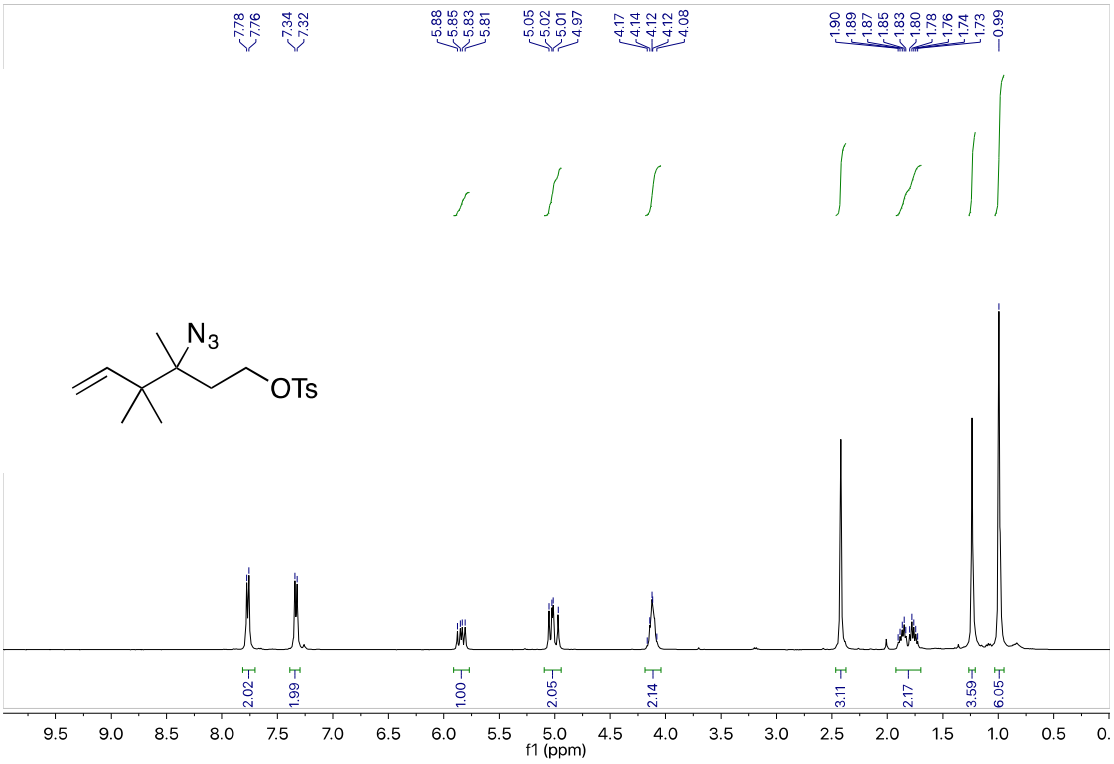
1.04
1.09
3.18
6.25
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.
f1 (ppm)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5.85
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4.12
4.08
1.90
1.89
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1.86
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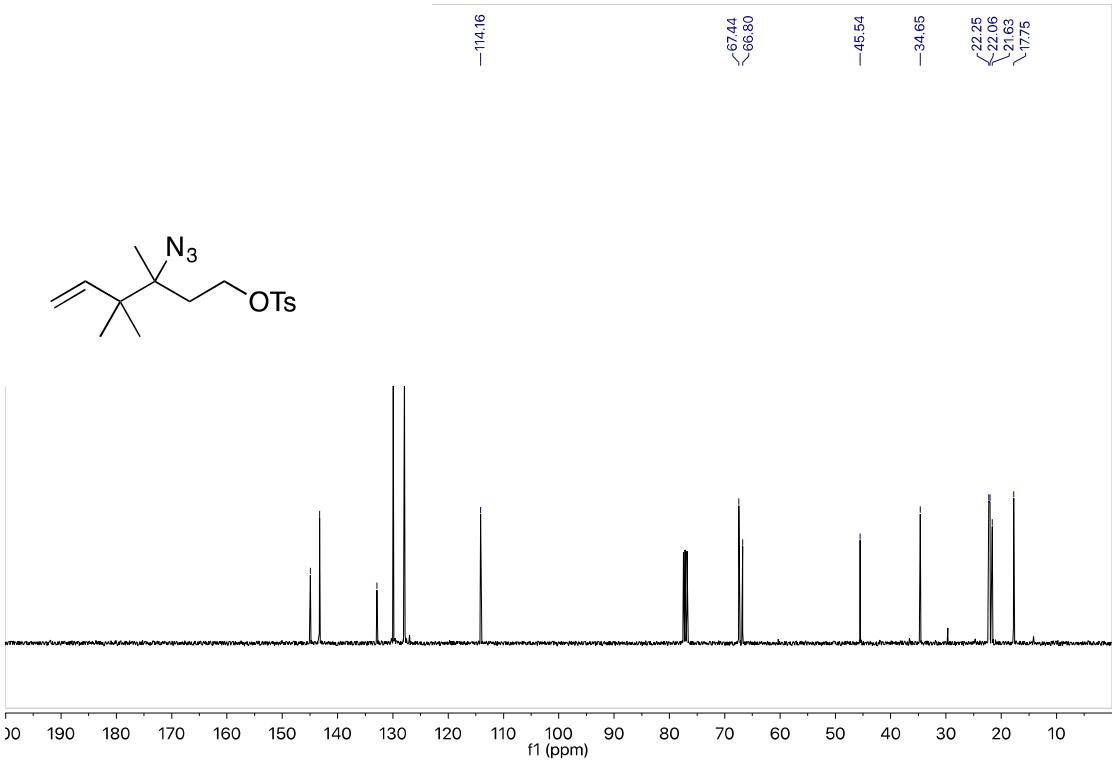
1.78
1.76
1.74
1.73
0.99
N3
OTs
2.02
1.99
1.00
2.05
2.14
3.11
2.17
3.59
6.05
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.
f1 (ppm)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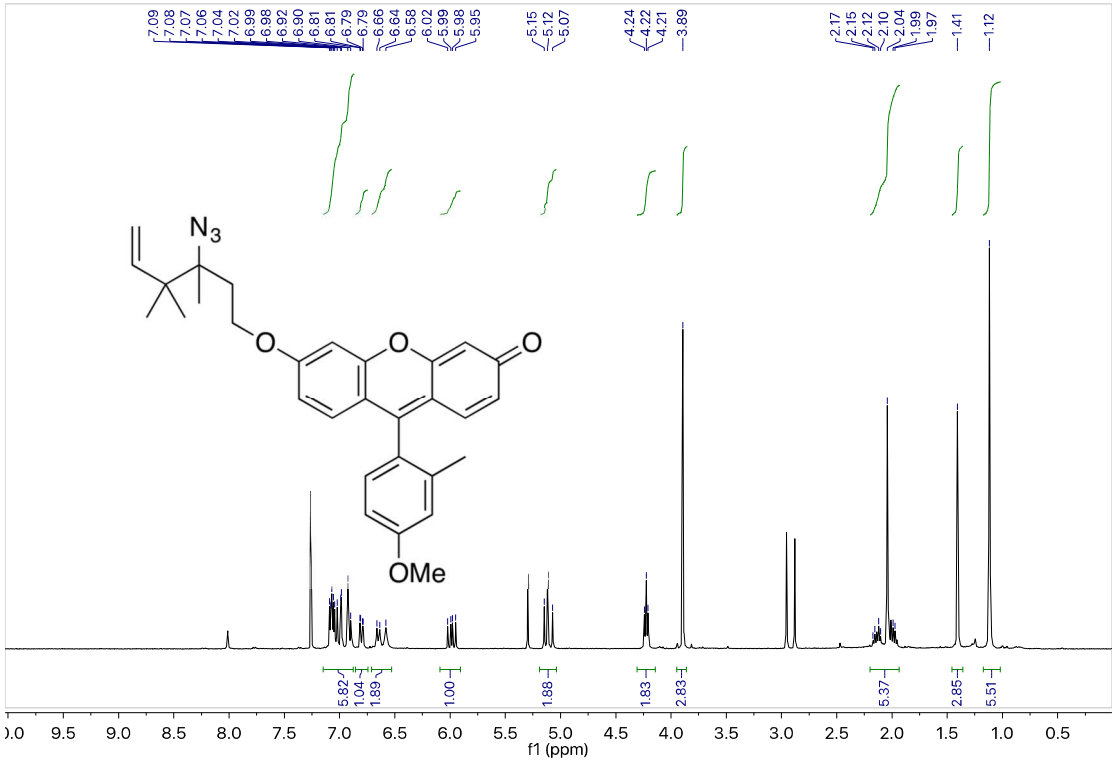
N3
OTs
f1 (ppm)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6.02
5.99
5.98
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5.07
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3.89
2.17
2.15
2.12
2.10
2.04
1.99
1.97
1.41
1.12
N3
O
O
O
OMe
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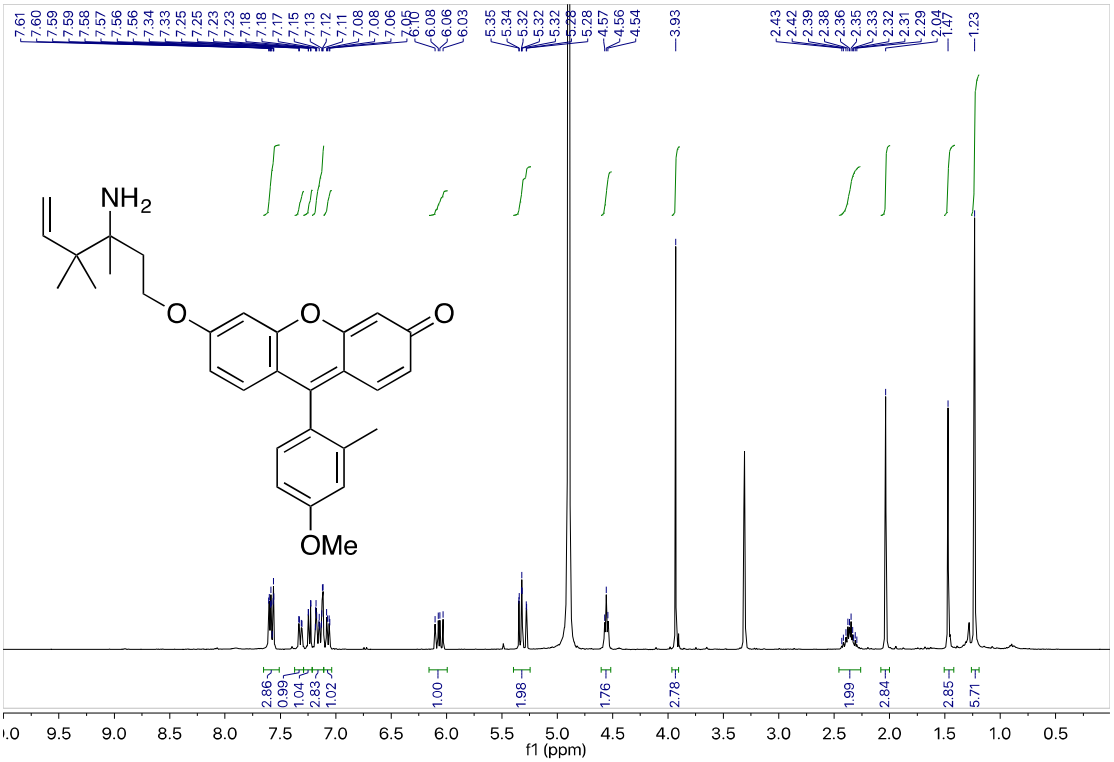
f1 (ppm)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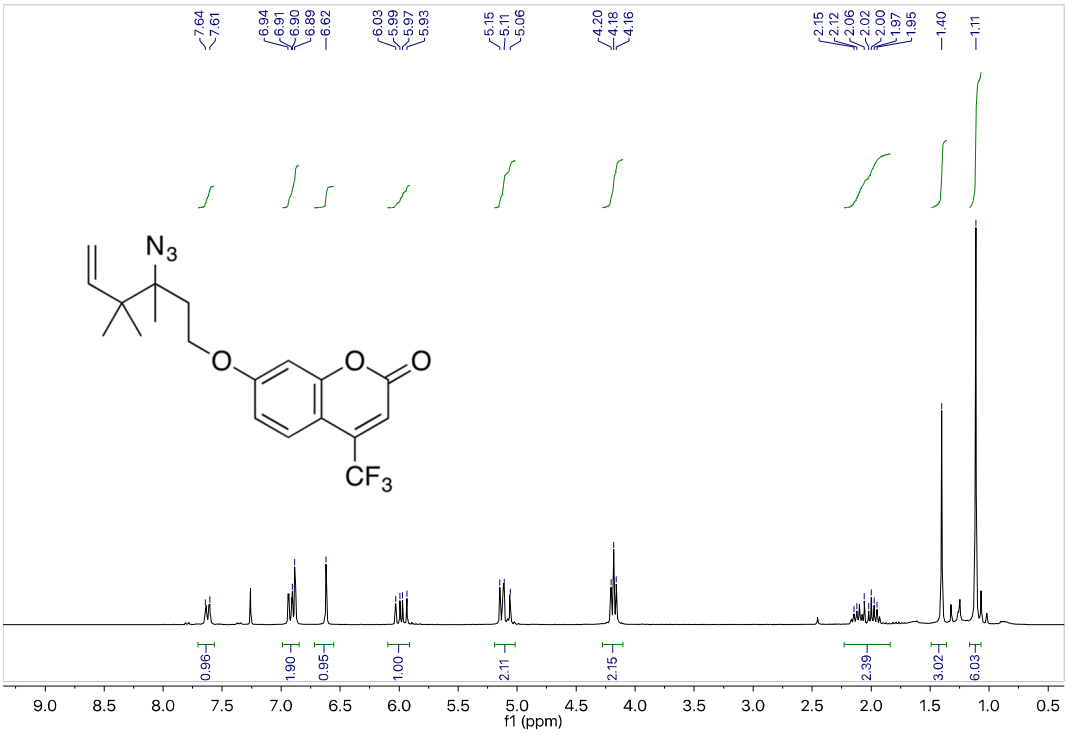
O
O
OMe
f1 (ppm)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CF3
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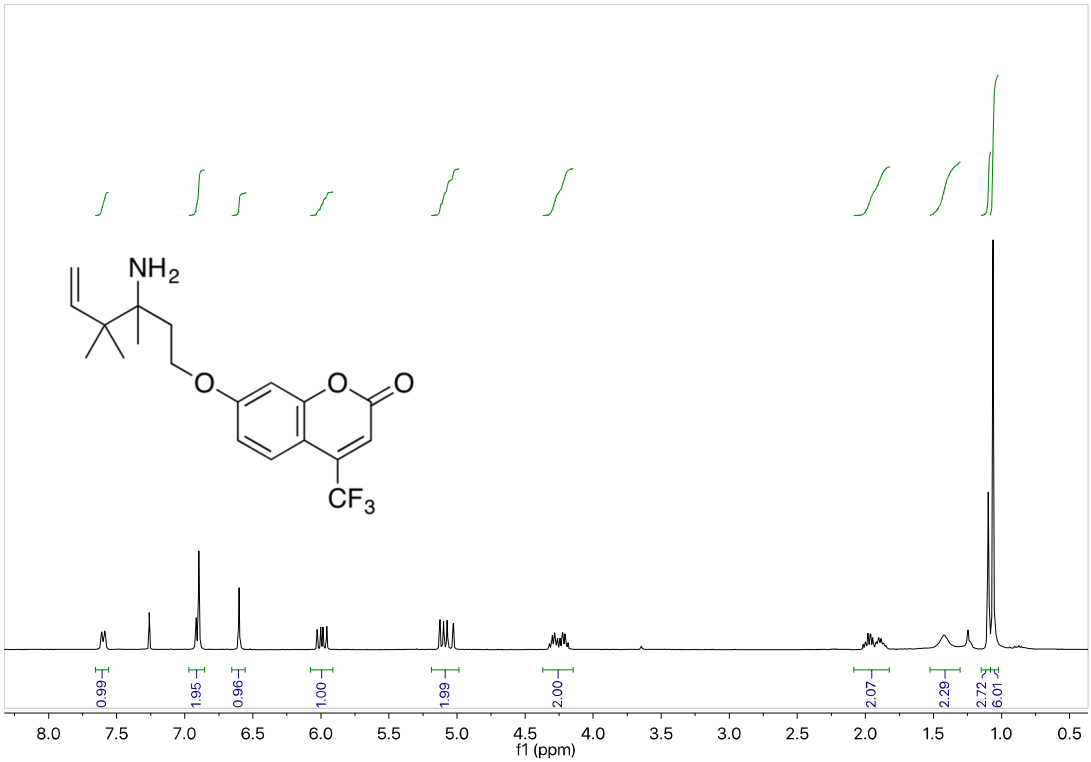
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6.0
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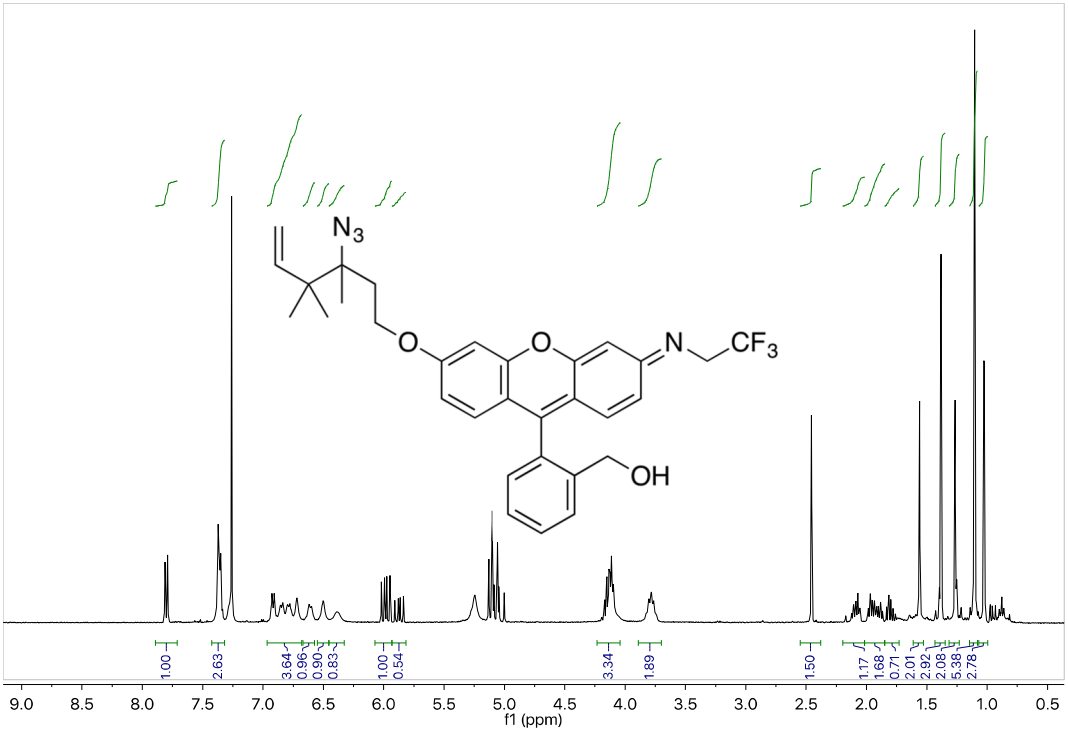
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7.0
6.5
6.0
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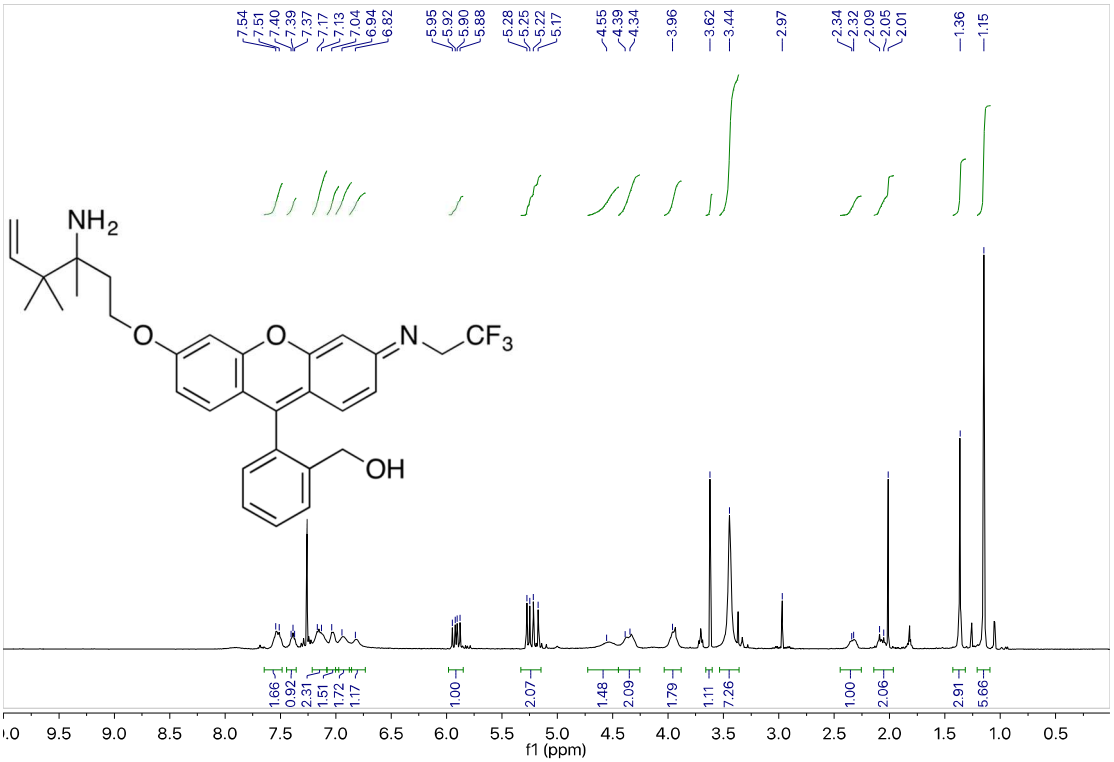
f1 (ppm)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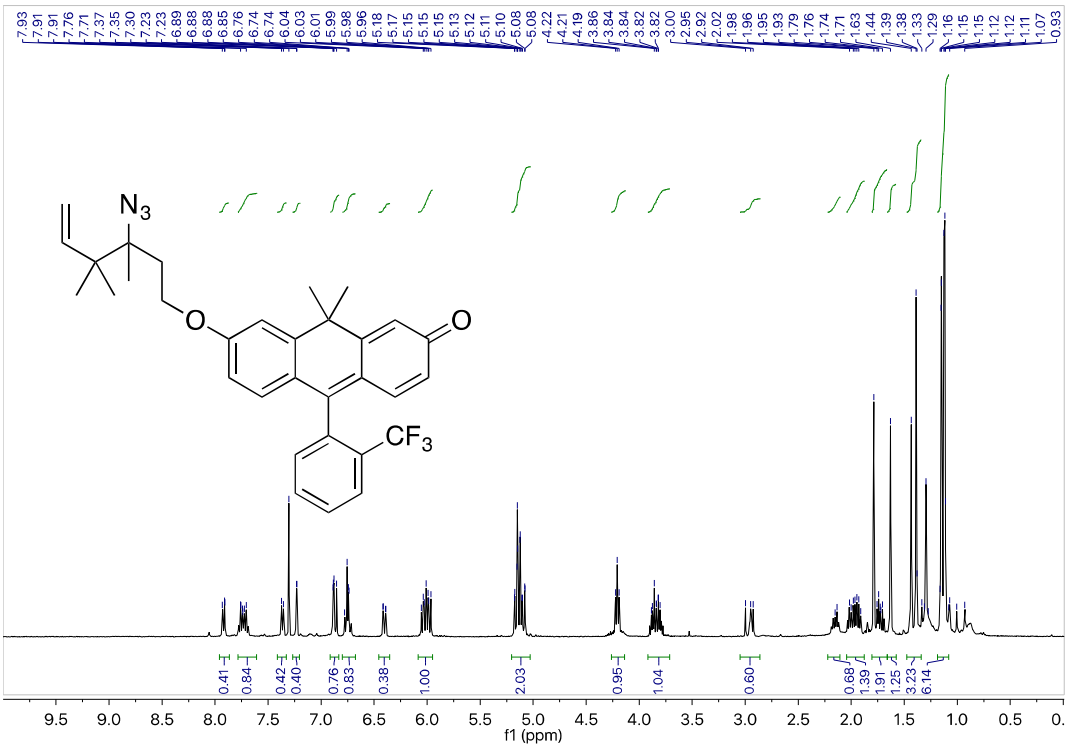
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O
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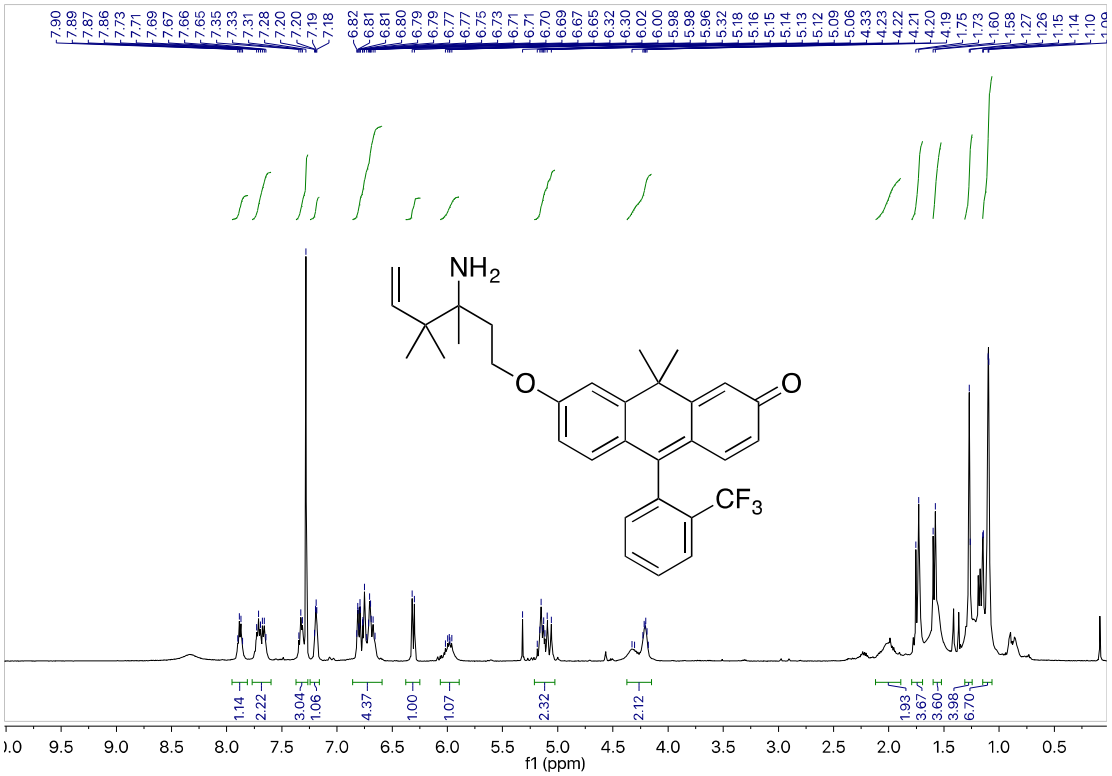
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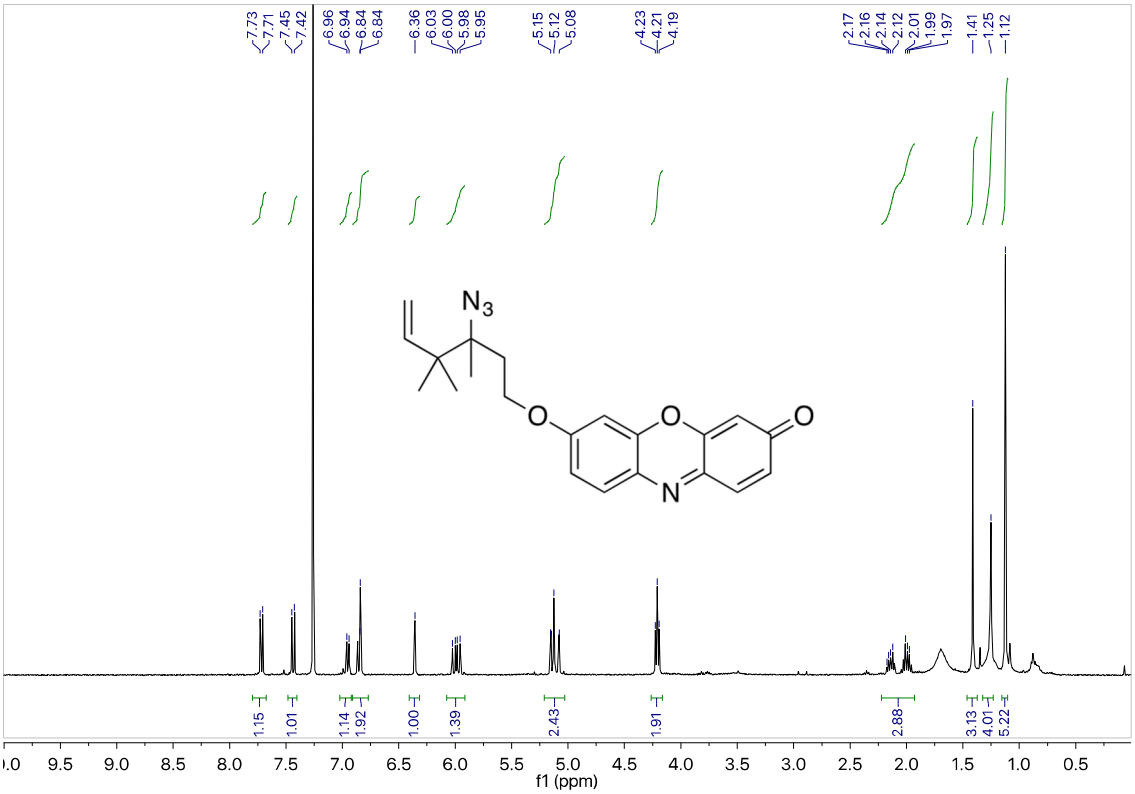
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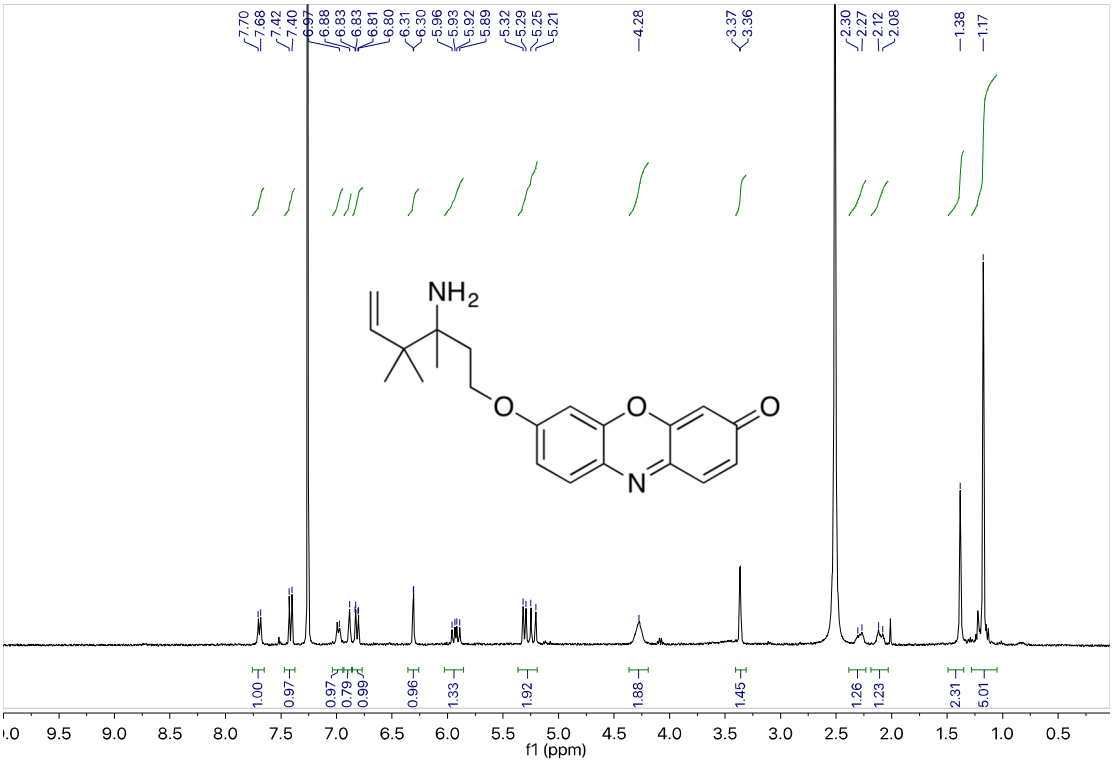
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5.96
5.93
5.92
5.89
5.32
5.29
5.25
5.21
4.28
3.37
3.36
2.30
2.27
2.12
2.08
1.38
1.17
NH2
O
O
O
N
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0.97
0.97
0.79
0.99
0.96
1.33
1.92
1.88
1.45
1.26
1.23
2.31
5.01
f1 (ppm)

Supplemental References:

1. Ran, F. A.; Hsu, P. D.; Wright, J.; Agarwala, V.; Scott, D. A.; Zhang, F. *Nat Protoc*. **2013**, *8*, 2281–2308.
2. Pontel, L. B.; Rosado, I. V.; Burgos-Barragan, G.; Garaycoechea, J. I.; Yu, R.; Arends, M. J.; Chandrasekaran, G.; Broecker, V.; Wei, W.; Liu, L.; Swenberg, J. A.; Crossan, G. P.; Patel, K. J. *Mol. Cell* **2015**, *60*, 177–188.