Supporting Information

DNA-Polyfluorophore Excimers as Sensitive Reporters for Esterases and Lipases

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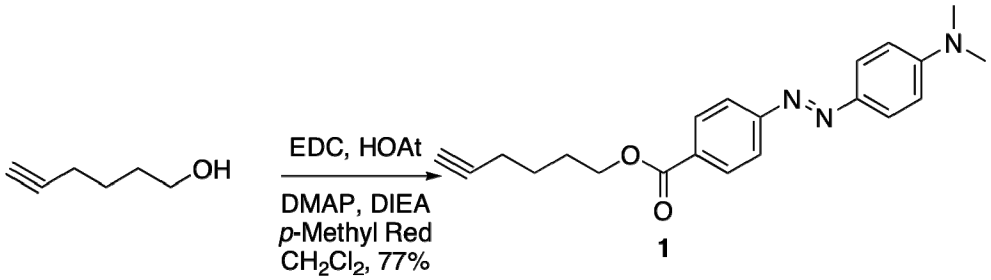
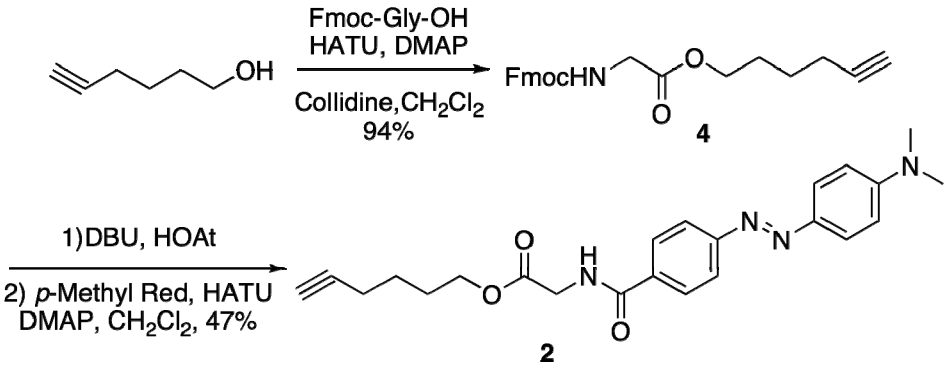
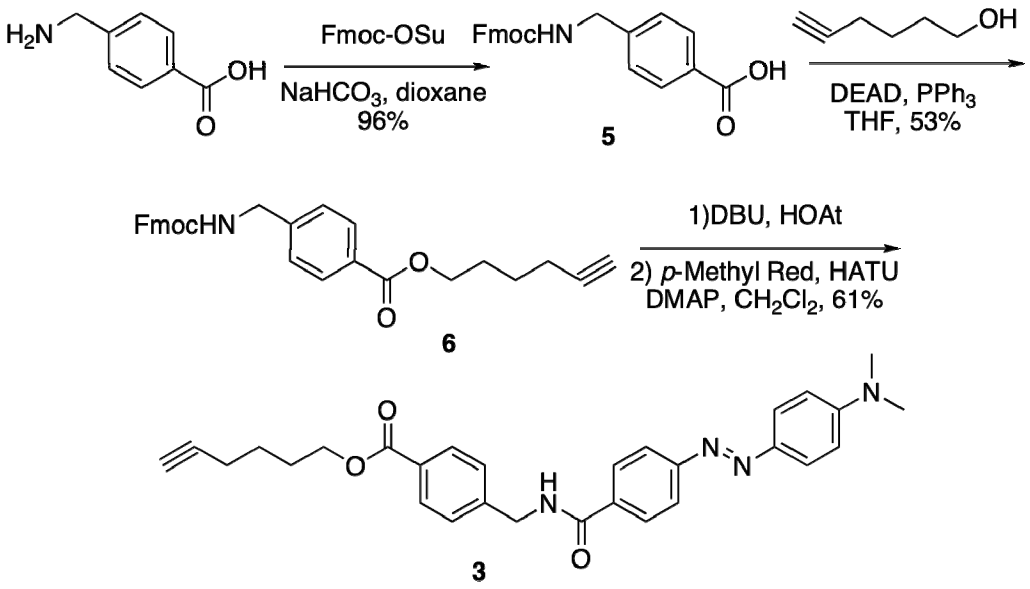


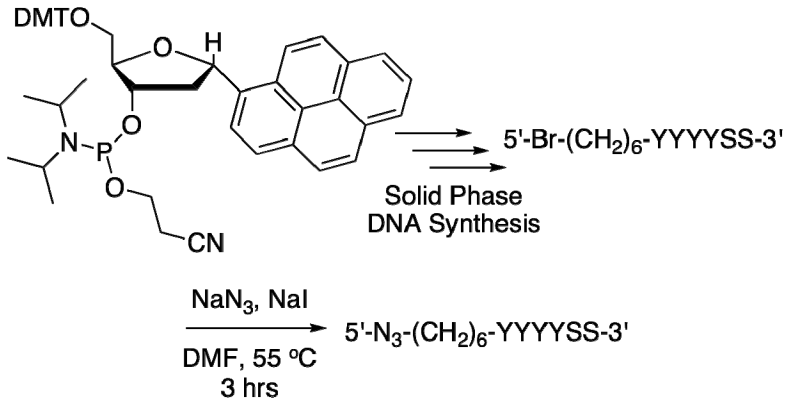
Scheme S2. Synthesis of Quencher **2**



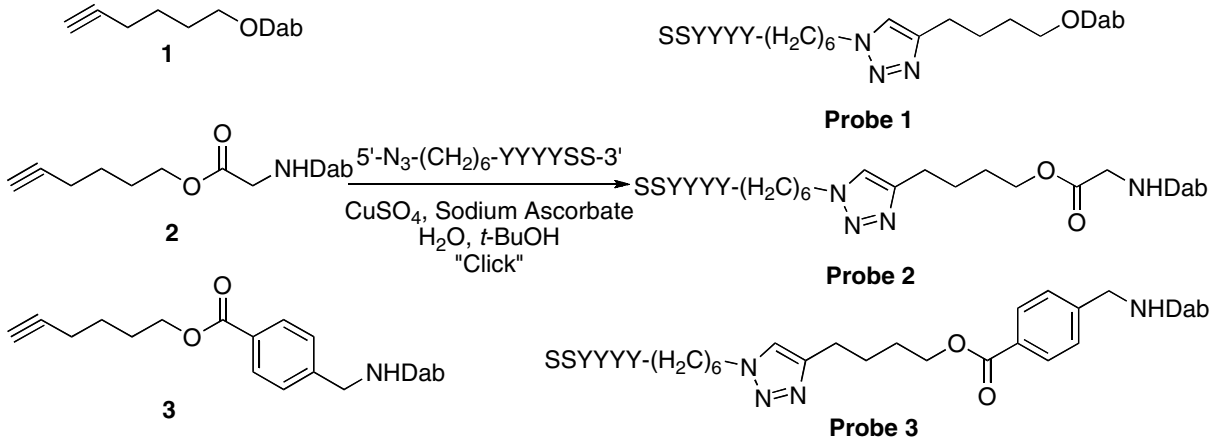
Scheme S3. Synthesis of Quencher **3**



Scheme S4. Solid phase synthesis of oligopyrene



Scheme S5. Synthesis of ODF-based fluorescent probes **1**, **2** and **3**.



Experimental Section

General. Amino acid derivatives, resins, and reagents were purchased. Unless otherwise indicated, all reactions were carried out under N_2 sealed from moisture. Anhydrous solvents were used directly from sealed bottles, which were stored under Ar. Brine (NaCl), $NaHCO_3$, and NH_4Cl refer to saturated aqueous solutions unless otherwise noted. Flash chromatography was performed on 32-63 μm silica gel with reagent grade solvents. NMR spectra were obtained at ambient temperature in $CDCl_3$ unless otherwise noted. ^1H and ^{13}C NMR spectra were obtained at 400 and 100 MHz, respectively, unless otherwise noted. Chemical shifts are reported in parts per million (ppm) downfield from tetramethylsilane (TMS). Data are reported as follows: chemical shift, multiplicity: singlet (s), doublet (d), triplet (t), quadruplet (q), quintuplet (qt), sextuplet (st), multiplet (m), coupling constants *J* in Hz, and integration. Enzymatic studies were performed in 1X pH 7.4 PBS buffer unless otherwise noted. The blank for probe only experiments contained the same volume of PBS buffer, or the same percentage of DMSO in PBS buffer. The blank for enzymatic reactions was the respective enzyme at the same concentration in the same solvent.

Organic Synthesis.

Hex-5-ynyl 4-((4-(dimethylamino)phenyl)diazo)benzoate, 1. *p*-Methyl red (627 mg, 2.32 mmol) was dissolved in CH_2Cl_2 (40 mL). EDC (670 mg, 3.50 mmol), HOAt (477 mg, 3.50 mmol), DMAP (20.5 mg, 0.168 mmol) and DIEA (2.00 mL, 11.5 mmol) were added, and the resulting solution was stirred at 0 °C for 20 min. Then 5-hexyn-1-ol (0.28 mL, 2.6 mmol) was added dropwise, and the solution was stirred at room temperature for 40 h. The solvent was removed in vacuo, and the resulting slurry was dissolved in EtOAc (100 mL). The organic layer was washed with H_2O (50 mL), $NaHCO_3$ (2 × 50 mL), and brine (50 mL), dried over Na_2SO_4, concentrated in vacuo, and purified with silica column chromatography (20 % EtOAc in hexanes). The product was obtained as a red solid (628 mg, 77 %). ^{1}H NMR: δ 8.14 (d, *J* = 8.8, 2H), 7.91 (d, *J* = 9.2, 2H), 7.86 (d, *J* = 8.6, 2H), 6.76 (d, *J* = 9.3, 2H), 4.37 (t, *J* = 6.4, 2H), 3.11 (s, 6H), 2.30 (dt, *J* = 7.1, 2.8, 2H), 1.99 (t, *J* = 2.6, 1H), 1.93 (m, 2H), 1.72 (m, 2H). ^{13}C NMR: δ 166.5, 156.1, 153.0, 143.8, 130.6, 130.4, 125.6, 122.1, 111.6, 84.0, 68.9, 64.7, 40.4, 27.9, 25.2, 18.3. HRMS (*m/z*): $[M + Na]^+$ calcd. for $C_{21}H_{23}N_3O_2Na$, 372.1688; found, 372.1687.

Hex-5-ynyl 2-(((9H-fluoren-9-yl)methoxy)carbonylamino)acetate, 4. Fmoc-Gly-OH (1.56 g, 5.24 mmol), HATU (2.19 g, 4.79 mmol), DMAP (30 mg, 0.24 mmol) and collidine (3.20 mL, 24.1 mmol) were dissolved in CH_2Cl_2 (50 mL), and stirred at 0 °C for 20 min. 5-Hexyn-1-ol (0.52 mL, 4.8 mmol) was added dropwise, and the solution was stirred at room temperature for 14 h. The solvent was removed in vacuo, and the resulting slurry was dissolved in EtOAc (100 mL). The organic layer was washed with 1 M HCl (2 × 50 mL), $NaHCO_3$ (2 × 50 mL), and brine (50 mL), dried over Na_2SO_4, concentrated in vacuo, and purified with silica column chromatography (CH_2Cl_2). A white solid was obtained (1.70 g, 94 %). ^{1}H NMR: δ 7.75 (d, *J* = 7.4, 2H), 7.59 (d, *J* = 7.6, 2H), 7.38 (app. t, *J* = 7.4, 2H), 7.30 (dt, *J* = 7.5, 1.3, 2H), 5.40 (t, *J* = 5.3, 1H), 4.39 (d, *J* = 7.2, 2H), 4.22 (t, *J* = 7.1, 1H), 4.17 (t, *J* = 6.5, 2H), 3.97 (d, *J* = 5.6, 2H), 2.21 (dt, *J* = 7.0, 2.7, 2H), 1.95 (t, *J* = 2.7, 1H), 1.76 (m, 2H), 1.67 (m, 2H). ^{13}C NMR: δ 170.1, 156.4, 143.9, 141.3, 127.8, 127.1, 125.1, 120.1, 83.8, 69.0, 67.2, 65.0, 47.1, 42.8, 27.6, 24.8, 18.1. HRMS (*m/z*): $[M + Na]^+$ calcd. for $C_{23}H_{23}NO_4Na$, 400.1525; found, 400.1517.

Hex-5-ynyl 2-(4-((4-(dimethylamino)phenyl)diazo)benzamido)acetate, 2. Fmoc–protected ester **4** (113 mg, 0.300 mmol) and HOAt (151 mg, 1.11 mmol) were dissolved in CH_2Cl_2 (15 mL). DBU (0.18 mL, 1.2 mmol) was added, and the solution was stirred at room temperature for 20 min. Then HATU (171 mg, 0.451 mmol), DMAP (2.1 mg, 0.017 mmol) and *p*-methyl red (98.5 mg, 0.366 mmol) were added, and the resulting solution was stirred at room temperature for 14 h. The solution was diluted with CH_2Cl_2 (20 mL), and washed with $NaHCO_3$ (20 mL). The aqueous layer was extracted with CH_2Cl_2 (3 × 20 mL). The organic layers were combined, dried over Na_2SO_4, and purified with silica column chromatography (1 % MeOH in CH_2Cl_2). A red solid (57 mg, 47 %) was obtained. ^{1}H NMR: δ 7.90 (m, 6H), 6.76 (d, *J* = 9.3, 2H), 6.70 (t, *J* = 4.5, 1H), 4.27 (d, *J* = 5.2, 2H), 4.25 (t, *J* = 6.7, 2H), 3.11 (s, 6H), 2.26 (dt, *J* = 7.0, 2.7, 2H), 1.98 (t, *J* = 2.7, 1H), 1.83 (m, 2H), 1.63 (m, 2H). ^{13}C NMR: δ 170.3, 167.1, 155.4, 153.0, 143.8, 133.8, 128.1, 125.6, 122.4, 111.6, 83.8, 69.1, 65.3, 42.1, 40.4, 27.7, 24.9, 18.2. HRMS (*m/z*): $[M + H]^+$ calcd. for $C_{23}H_{27}N_4O_3$, 407.2083; found, 407.2075.

Hex-5-ynyl 4-((((9*H*-fluoren-9-yl)methoxy)carbonylamino)methyl)benzoate, 6. Fmoc-aminomethyl *p*-benzoic acid **5** was synthesized based on the previous reported method[1] with small modification. 4-Amino methyl benzoic acid (346 mg, 2.29 mmol) was dissolved in

saturated $NaHCO_3$ (8 mL), and stirred at 0 °C for 10 min. Fmoc-OSu (857 mg, 2.54 mmol) was dissolved in dioxane (5 mL), and added dropwise to the solution. The resulting solution was stirred at room temperature for 14 h. Then the solution was acidified to pH 1 with 1 M HCl to yield white precipitates. The resulting solid was filtered, washed with H_2O (4 × 5 mL) and hexanes (4 × 5 mL), dried in vacuo, collected and used without further purification. The product was obtained as a white solid (815 mg, 96 %). ^{1}H NMR (DMSO-d^6): δ 7.89 (m, 5H), 7.70 (d, J = 7.6, 2H), 7.42 (t, J = 7.4, 2H), 7.33 (m, 4H), 4.37 (d, J = 6.9, 2H), 4.24 (m, 3H). Fmoc-aminomethyl *p*-benzoic acid **5** (334 mg, 0.895 mmol) and triphenyl phosphine (258 mg, 0.985 mmol) were dissolved in THF (10 mL); then 5-hexyn-1-ol (97 μL, 0.894 mmol) was added, followed by DEAD (0.45 mL, 0.99 mmol). The resulting solution was stirred at room temperature for 30 min, and then diluted with CH_2Cl_2 (30 mL). The organic layer was washed with NH_4Cl (2 × 20 mL), $NaHCO_3$ (2 × 20 mL), and brine (20 mL), dried over Na_2SO_4, concentrated in vacuo, and purified with silica column chromatography (20 % EtOAc in hexanes). A white solid was obtained (217 mg, 53 %). ^{1}H NMR: δ 7.96 (d, J = 8.2, 2H), 7.73 (d, J = 7.6, 2H), 7.56 (d, J = 7.5, 2H), 7.37 (t, J = 7.4, 2H), 7.27 (m, 4H), 5.37 (t, J = 6.0, 1H), 4.44 (d, J = 6.7, 2H), 4.36 (d, J = 6.3, 2H), 4.32 (t, J = 6.4, 2H), 4.17 (t, J = 6.8, 1H), 2.26 (dt, J = 7.0, 2.6, 2H), 1.98 (t, J = 2.5, 1H), 1.88 (m, 2H), 1.67 (app. qt, J = 7.3, 2H). ^{13}C NMR: δ 166.4, 156.6, 143.84, 143.79, 141.3, 129.9, 129.4, 127.7, 127.2, 127.1, 125.0, 120.0, 83.9, 68.9, 66.7, 64.5, 47.3, 44.6, 27.8, 25.1, 18.1. HRMS (*m/z*): $[M + Na]^+$ calcd. for $C_{29}H_{27}NO_4Na$, 476.1838; found, 476.1827.

Hex-5-ynyl 4-((4-((4-(dimethylamino)phenyl) diazo)benzamido)methyl) benzoate, 3. Fmoc–protected ester **6** (217 mg, 0.479 mmol) and HOAt (252 mg, 1.85 mmol) were dissolved in CH_2Cl_2 (25 mL). DBU (0.30 mL, 2.0 mmol) was added, and the solution was stirred at room temperature for 20 min. Then HATU (276 mg, 0.725 mmol), DMAP (3.1 mg, 0.025 mmol) and *p*-methyl red (156 mg, 0.578 mmol) were added, and the resulting solution was stirred at room temperature for 14 h. The solution was diluted with CH_2Cl_2 (40 mL), and washed with $NaHCO_3$ (30 mL). The aqueous layer was extracted with CH_2Cl_2 (3 × 20 mL). The organic layers were combined, dried over Na_2SO_4, and purified with silica column chromatography (1 % MeOH in CH_2Cl_2). A red solid (141 mg, 61 %) was obtained.^{1}H NMR: δ 8.00 (d, J = 8.3, 2H), 7.88 (m, 6H), 7.41 (d, J = 8.6, 2H), 6.74 (m, 3H), 4.70 (d, J = 6.0, 2H), 4.33 (t, J = 6.4, 2H), 3.09 (s, 6H), 2.27 (dt, J = 7.0, 2.7, 2H), 1.98 (t, J = 2.7, 1H), 1.89 (m, 2H), 1.69 (m, 2H). ^{13}C NMR: δ 167.2, 166.4, 155.3, 152.9, 143.7, 143.6, 134.1, 130.1, 129.7, 128.0, 127.7, 125.6, 122.4, 111.6, 84.0, 68.9, 64.6, 43.9, 40.4, 27.8, 25.1, 18.2. HRMS (*m/z*): $[M + Na]^+$ calcd. for $C_{29}H_{30}N_4O_3Na$, 505.2216; found, 505.2213.

Oligodeoxyfluorosides Synthesis. 5'-DMT 3'-cyanoethyl α-pyrene phosphoramidite monomer was prepared according to published methods.[2, 3] The pyrene phosphoramidite was dissolved in CH_3CN:CH_2Cl_2 (1:1), while C3 spacer phosphoramidite and 5'-bromohexyl phosphoramidite (Glen Research) were dissolved in CH_3CN at 0.1 M concentration. Test oligonucleotide synthesis on DNA synthesizer showed that good coupling yields (95-99%) could be achieved for all monomers. The ODF, YYYY, was synthesized on an Applied Biosystems 394 DNA/RNA synthesizer, using 3'-phosphate CPG column on 1 μmol scale with DMT-on method. Coupling of each monomer used standard 3' to 5' cyanoethyl phosphoramidite chemistry with extended coupling time (999 s). After the solid phase DNA synthesis, the CPG solid support was transferred to a vial, and a solution of NaN_3 (13 mg) and NaI (30 mg) in DMF (2.0 mL) was

added. The reaction vial was heated at 55 °C for 4 h and then cooled to room temperature. The solid support was washed with DMF (3 × 2.0 mL) and H_2O (3 × 2.0 mL), and dried in vacuo. Cleavage of the ODF from solid support was accomplished using saturated NH_4OH for 17 h at room temperature, then for another 3 h at 37 °C to ensure complete cleavage. Crude ODF was purified on a semi-preparative Phenomenox Juniper C5 column (250 × 10 mm, 5 μm) using a gradient of TEAA buffer (50 mM, pH 7.0) in H_2O and CH_3CN. The purified 5'-azido ODF was obtained as a white solid and stored under Ar at –20 °C. MALDI-MS (*m/z*): $[M + H]^+$ calcd. for $C_{96}H_{97}N_3O_{32}P_7$, 2020.42; found, 2019.96.

Huisgen-Sharpless conjugation. The alkyne-quencher was dissolved in DMSO (10 mM), and ODF (0.2-0.5 mM), $CuSO_4$ (100 mM) and sodium ascorbate (100 mM) were dissolved in H_2O. For each conjugation reaction, ODF (70 μL) and sodium ascorbate (40 μL), *t*-BuOH (150 μL) and alkyne quencher (30 μL) were added subsequently. After the addition of $CuSO_4$ (40 μL), the reaction vial was sealed and protected from air and light, and shaken at room temperature for 24 h. Then the reaction mixture was purified with HPLC using a semi-preparative Phenomenox Juniper C5 column (250 × 10 mm, 5 μm) with a gradient of TEAA buffer (50 mM, pH 7.0) in H_2O and CH_3CN. The purified probe was obtained as an orange solid and stored under Ar at –20 °C. **Probe 1:** MALDI-MS (*m/z*): $[M + H]^+$ calcd. for $C_{117}H_{120}N_6O_{34}P_7$, 2369.60; found, 2369.94. **Probe 2:** MALDI-MS (*m/z*): $[M + H]^+$ calcd. for $C_{119}H_{123}N_7O_{35}P_7$, 2426.62; found, 2426.59. **Probe 3:** MALDI-MS (*m/z*): $[M + H]^+$ calcd. for $C_{125}H_{127}N_7O_{35}P_7$, 2502.65; found, 2504.66.

Enzyme screening. The concentrations of probes were estimated by pyrene UV absorbance at 342 nm (ε = 47,000 cm^{-1} M^{-1}).[4] The probes were dissolved in PBS buffer to prepare a stock solution of 20 μM. Esterases and lipases were dissolved in PBS buffer to prepare 1.0 mg/mL stock solution. The enzyme screening experiments were performed in a 96-well black quartz microplate with clear bottom on Flexstation II 384 microplate reader at 25 °C. Each reaction well contained 0.5 μM of probe and 30 μg/mL of enzyme in PBS buffer (100 μL). All samples were excited at 340 nm, and fluorescence emission was recorded at 480 nm with 420 nm cut-off using bottom read mode.

Fluorescence assay. Fluorescence studies were performed on a Jobin Yvon-Spex Fluorolog 3 spectrophotometer with porcine liver esterase (PLE, Sigma E2884) at ambient temperature. For ODF-based probes, samples were excited at 340 nm. Fluorescence spectra were recorded from 355 to 600 nm with 1 nm increment and 1 s integration time, and kinetic curve were obtained by monitoring fluorescence change at 480 nm. For fluorescein diacetate, samples were excited at 490 nm. Fluorescence spectra were recorded from 495 to 700 nm in 5 % DMSO in PBS buffer (0.10 μM probe, 10 μg/mL PLE), and kinetic curves were obtained by monitoring fluorescence change at 514 nm. For stability assay, both ODF-based **probe 1** and fluorescein diacetate were dissolved in 1 % DMSO in PBS buffer at 0.10 μM concentration.

Cell uptake studies.
Cell Culture. HeLa CCL-2 cells (ATCC) were maintained in Dulbelcco's modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin (all reagents from Invitrogen) in a humidified atmosphere at 37°C with 5% CO_2.

Cellular uptake and colocalization. Cells were plated in chambered coverglass (0.5 ml medium/chamber) and allowed to reach 60% confluency in 1-2 days. The growth medium was removed and new growth medium containing either 5 mM of probe **1** or probe **2** was added to the cells and incubated overnight at 37 °C under 5% CO_2. Cells were then washed thrice with PBS. Incubation with wheat germ agglutinin Alexa Fluor 633 conjugate at 5 μg/mL for 10 min was then carried out in studies to check for ODF internalization. Cells were rinsed three more times with PBS and fresh media was added before imaging. Cells without wheat germ agglutinin colabeling were visualized directly without a wash step.

Fluorescence imaging. Live HeLa cells were visualized directly in chambered coverglass using a Delta Vision deconvolution microscopy system. Images were acquired as stacks of optical sections with an interval of 0.2 μm viewed through a 63X oil objective. Hg lamp light source was used with appropriate filters to visualize the ODFs and AlexaFluor 633. Image acquisition was performed at the Cell Sciences Imaging Facility of Beckman Center for Molecular and Genetic Medicine, Stanford University Medical Center.

Enzyme List

Esterases

PLE: Esterase from porcine liver, suspension in 3.2 M $(NH_4)_2SO_4$, 1.22 mg/mL, 171.1 U/mg protein (Sigma E2884)
HLE: Esterase from hog liver, 165 U/mg (Fluka 46058)
SCE: Esterase from *Saccharomyces cerevisiae*, 2.2 U/g (Fluka 46071)
HrLE: Esterase from horse liver, 0.5 U/mg (Fluka 46069)
MME: Esterase from *Mucor miehei*, 1.08 U/mg (Fluka 46059)
CLE: Esterase from *Candida lipolytica*, 0.10 U/mg (Fluka 46056)
BSE: Esterase from *Bacillus stearothermophilus*, 0.47 U/mg (Fluka 46051)
BE: Esterase from *Bacillus species*, 0.11 U/mg (Fluka 46062)
SDE: Esterase from *Streptomyces diastatochromgenes*, recombinant from *E. coli*, 31.8 U/mg (Fluka 78042)
PFE: Esterase from *Pseudomonas fluorescens*, recombinant from *E. coli*, 4.8 U/mg (Fluka 75742)
ROE: Esterase from *Rhizopus oryzae*, 66.7 U/mg (Fluka 79208)

Lipases

AL: Lipase from *Aspergillus*, 0.2 U/mg (Fluka 84205)
CAL: Lipase from *Candida Antarctica*, 1.51 U/mg (Fluka 65986)
CCL: Lipase from *Candida cylindracea*, 4.01 U/mg (Fluka 62316)
MML: Lipase from *Mucor miehei*, 1.19 U/mg (Fluka 62298)
PCL: Lipase from *Pseudomonas cepacia*, 46.2 U/mg (Fluka 62309)
PFL: Lipase from *Pseudomonas fluorescens*, 35.2 U/mg (Fluka 95608)
RAL: Lipase from *Rhizopus arrhizus*, 10.5 U/mg (Fluka 62305)
RNL: Lipase from *Rhizopus niveus*, 4.49 U/mg (Fluka 62310)
HPL: Lipase from hog pancreas, 30.1 U/mg (Fluka 62300)
ANL: Lipase from *Aspergillus niger*, 184 U/g (Fluka 62301)

References

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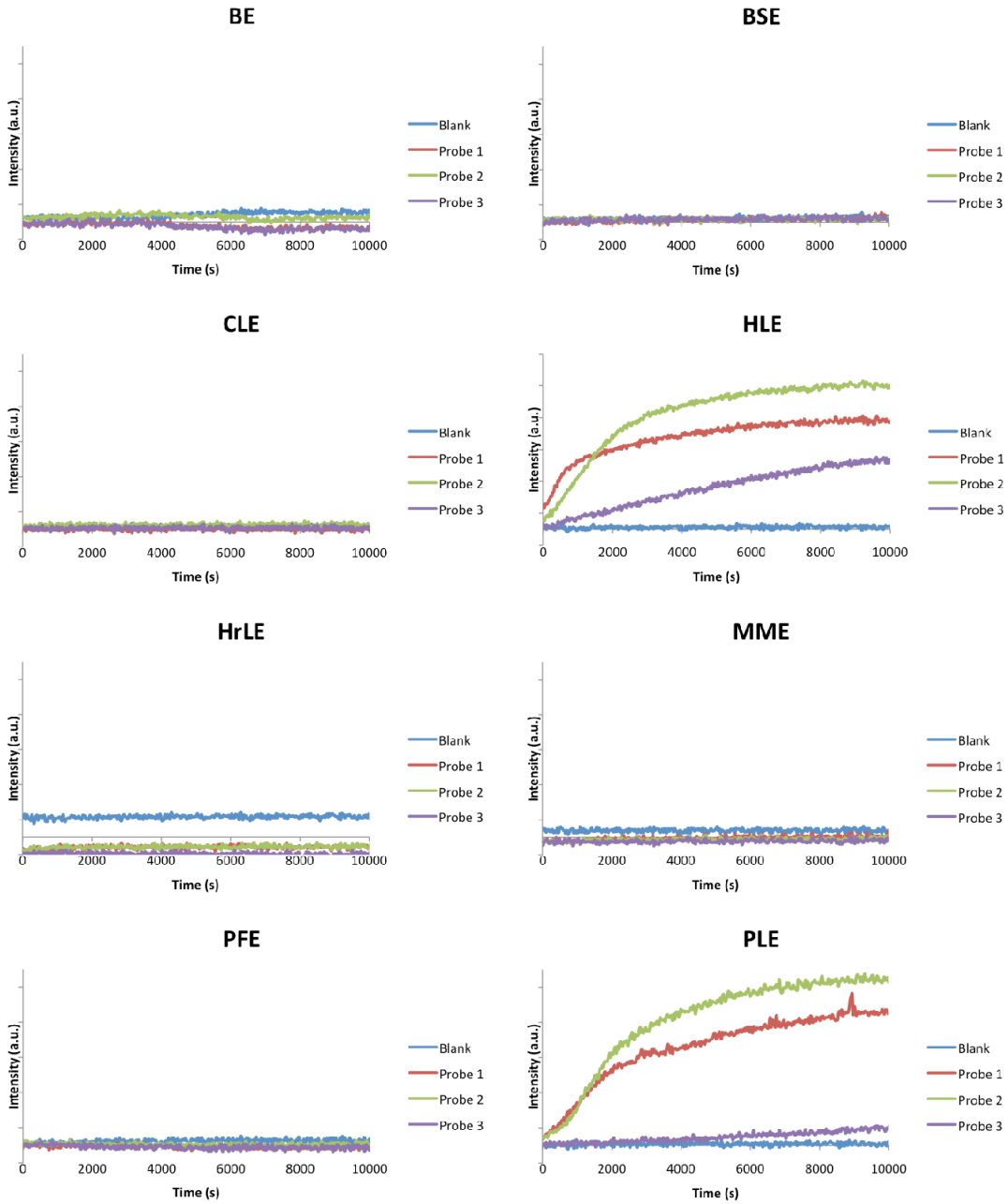
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BSE
CLE
HLE
HrLE
MME
PFE
PLE
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Probe 2
Probe 3

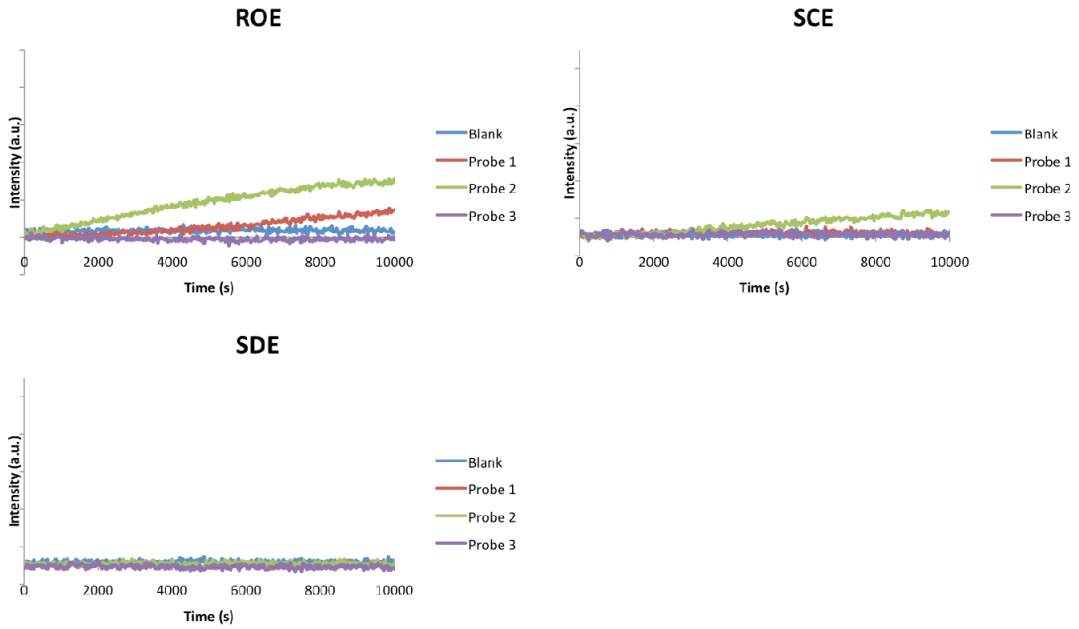
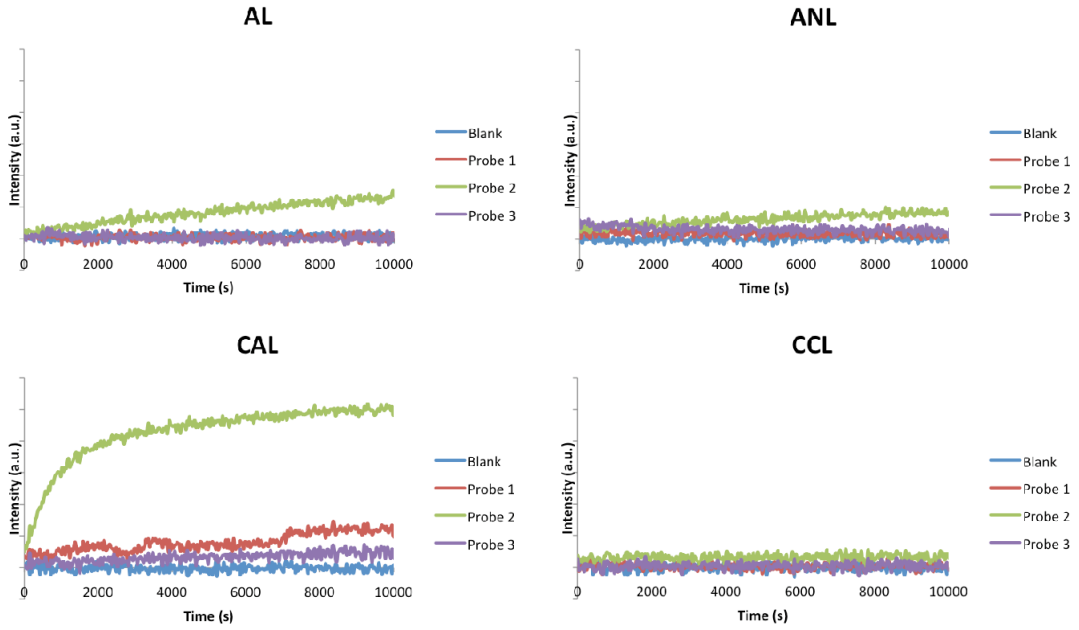


Figure S1. Esterase screening results. Each reaction well contained 0.5 μM probe and 30 μg/mL enzyme in pH 7.4 PBS buffer at 25 °C. All samples were excited at 340 nm, and fluorescent signals were monitored at 480 nm. Blank was 30 μg/mL of the respective esterase in pH 7.4 PBS buffer.



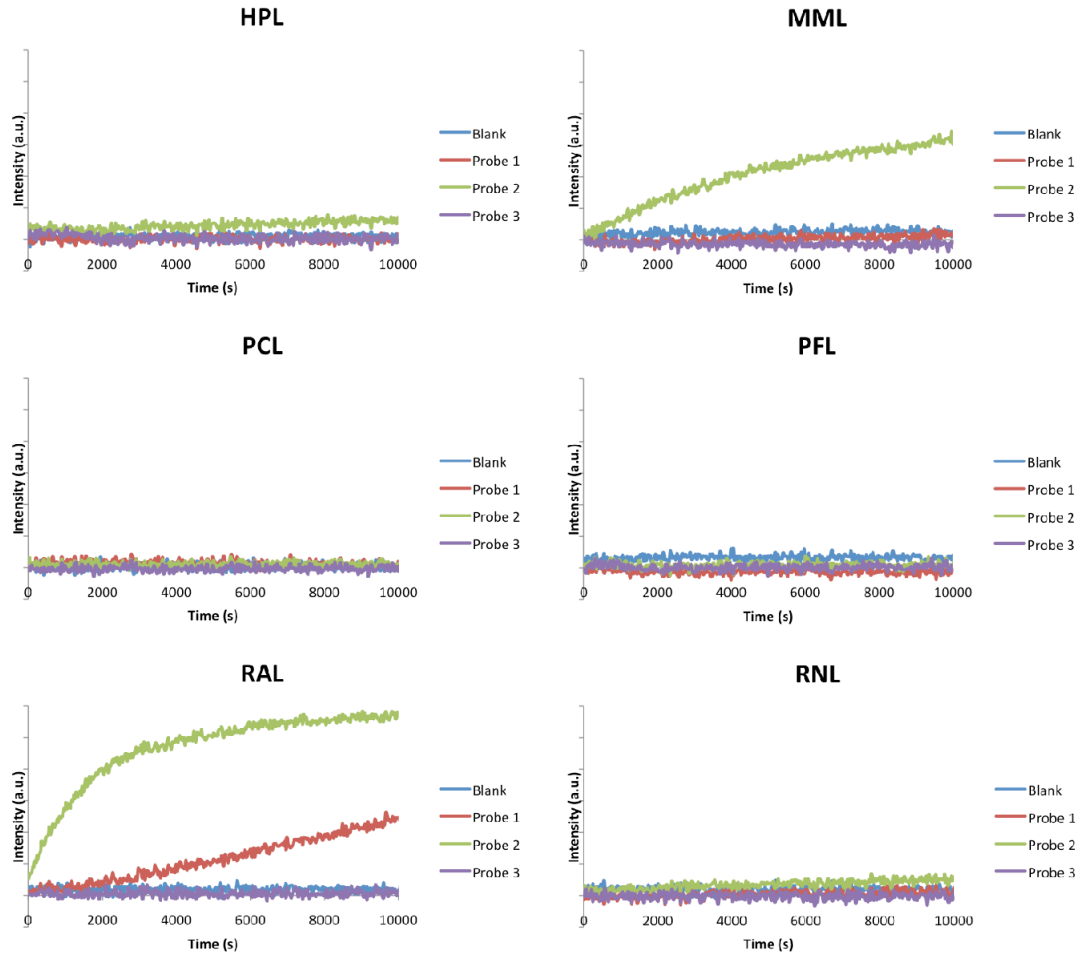


Figure S2. Lipase screening results. Each reaction well contained 0.5 μM probe and 30 μg/mL enzyme in pH 7.4 PBS buffer at 25 °C. All samples were excited at 340 nm, and fluorescent signals were monitored at 480 nm. Blank was 30 μg/mL of the respective lipase in pH 7.4 PBS buffer.

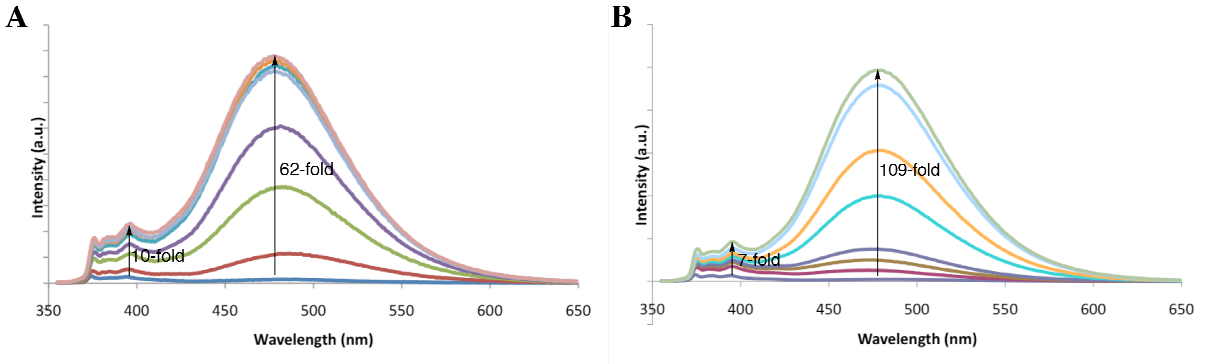


Figure S3. Fluorescence turn-on of probe **1** with PLE. Enzymatic fluorescence assay was performed in pH 7.4 PBS buffer at room temperature. (A) 0.13 μM probe with 12 μg/mL of PLE; (b) 1.1 μM probe with 102 μg/mL of PLE. The time course was measured over 5 h.

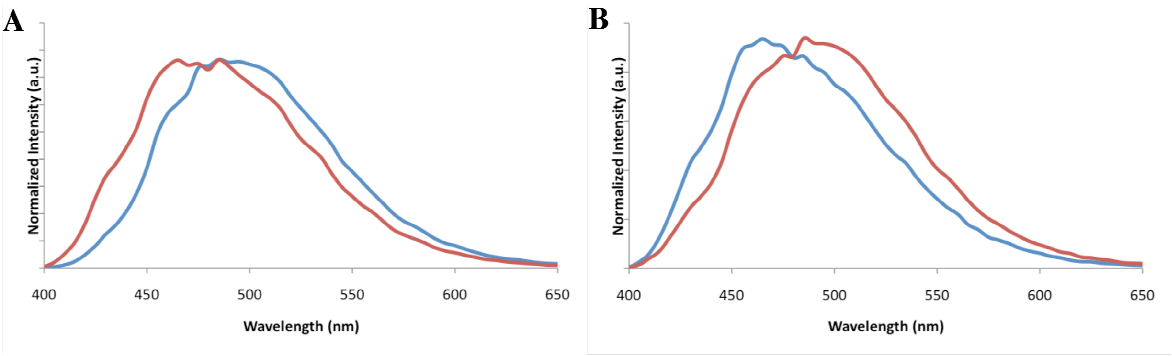


Figure S4. Normalized Fluorescent spectra at different PLE–Probe **1** ratios. Fluorescent spectra were recorded after 2 h of reaction in 1X PBS buffer (pH 7.4) at 25 °C in 96-well clear bottom quartz microplate on a fluorescence microplate reader with a cut-off wavelength at 420 nm using bottom-read mode. (A) With 0.8 µM probe. Blue line: 12 µg/mL of PLE; red line 122 µg/mL of PLE. (B) With 30 µg/mL of PLE. Blue line: 0.16 µM of probe **1**; red line: 1.6 µM of probe **1**.

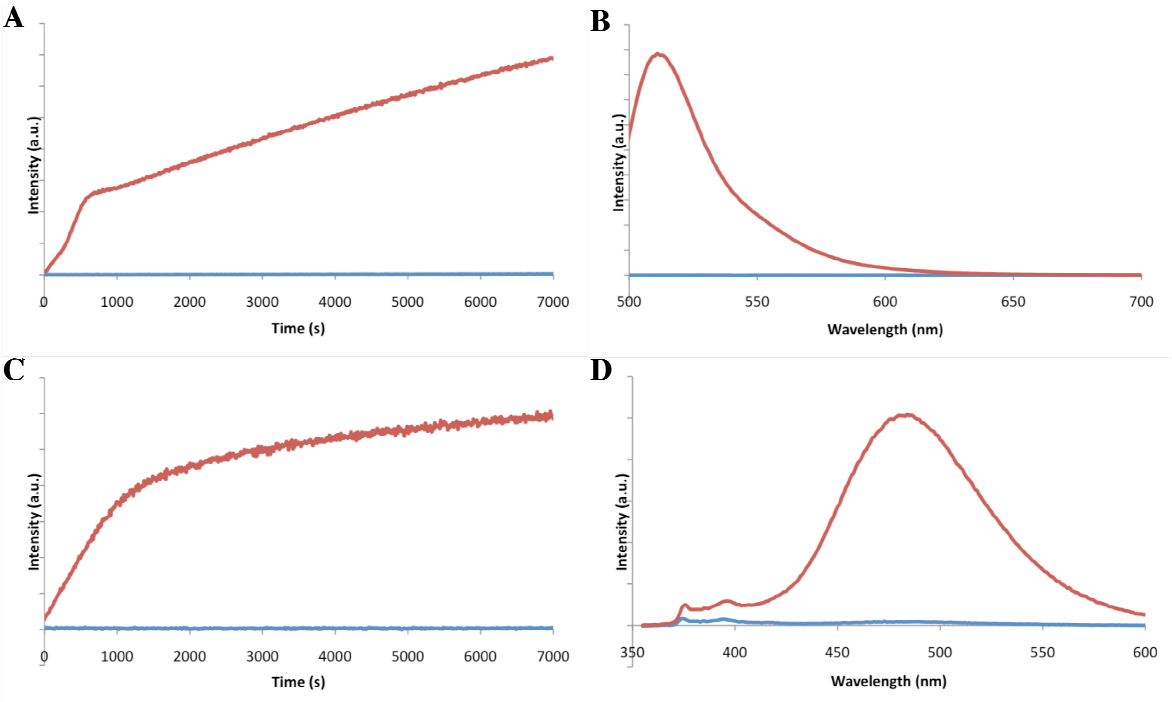


Figure S5. Enzymatic assay of fluorescein diacetate and probe **1.** Assay was performed with 0.1 µM of sensor, 10 µg/mL of PLE in 5% DMSO in pH 7.4 PBS buffer at room temperature. FDA was excited at 490 nm, and kinetic data was obtained at 514 nm. Probe **1** was excited at 340 nm, and kinetic data was obtained at 480 nm. (A) Kinetic plot of FDA; (B) full fluorescence spectrum of FDA after 2 h; (C) kinetic plot of probe **1**; (D) full fluorescence spectrum of probe **1** after 2 h. Red line: mixture of sensor and PLE; blue line: blank of sensor only.

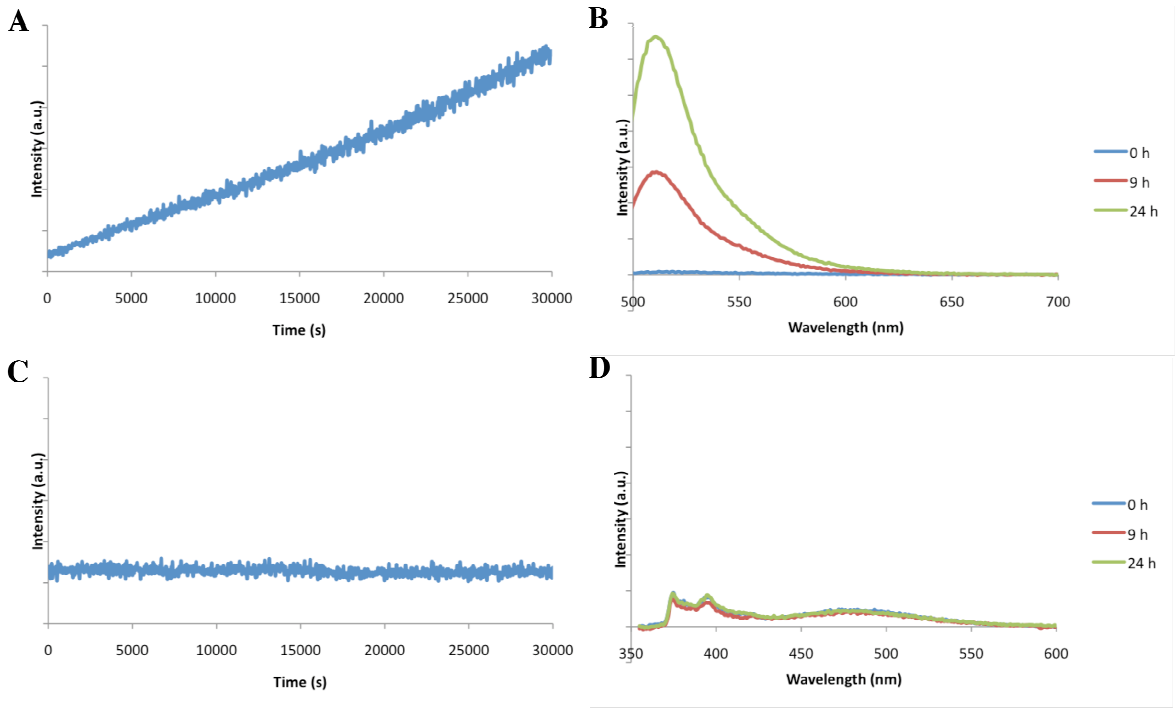


Figure S6. Fluorescence data comparing stability of FDA and probe **1**. The stability test was performed at 0.1 μM FDA sensor concentration, in 1% DMSO in PBS buffer at room temperature. FDA was excited at 490 nm, and kinetic data was obtained at 514 nm. Probe **1** was excited at 340 nm, and kinetic data was obtained at 480 nm. (A) Kinetic plot of FDA; (B) full fluorescence spectrum of FDA after 24 h; (C) kinetic plot of probe **1**; (D) full fluorescence spectrum of probe **1** after 24 h.

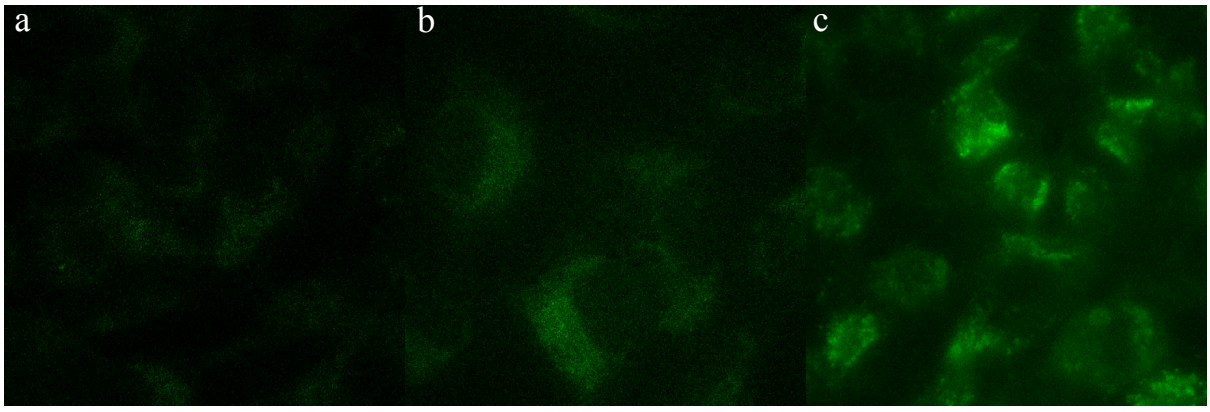
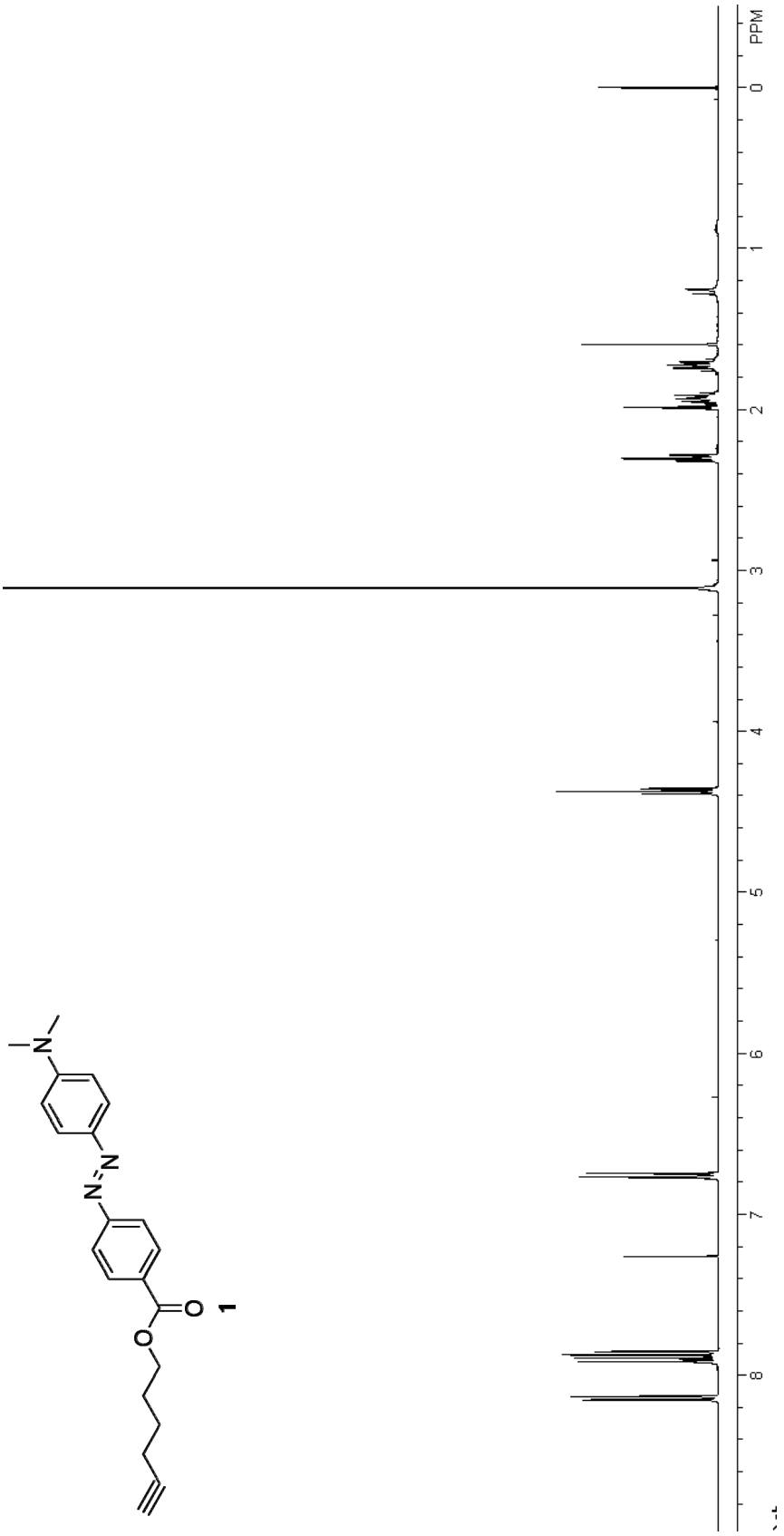


Figure S7. Epifluorescence images of cellular experiments without washing. HeLa cells were incubated with 5 mM of probe **1** or probe **2** at 37 °C under 5% CO_2 for 24 h. Pictures were taken without washing with PBS buffer. a) Negative control (HeLa cells only); b) incubated with probe **1**; c) incubated with probe **2**.



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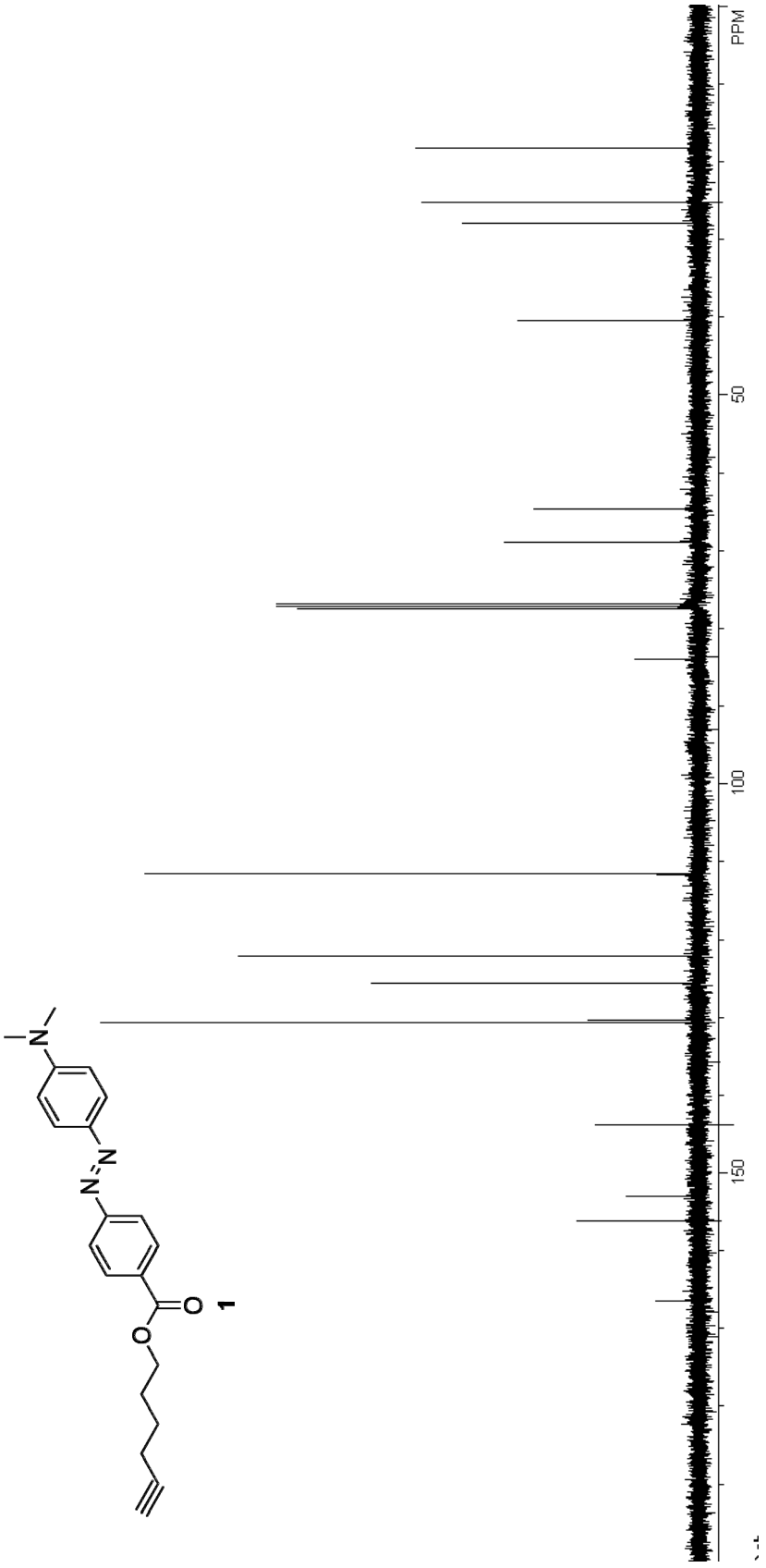
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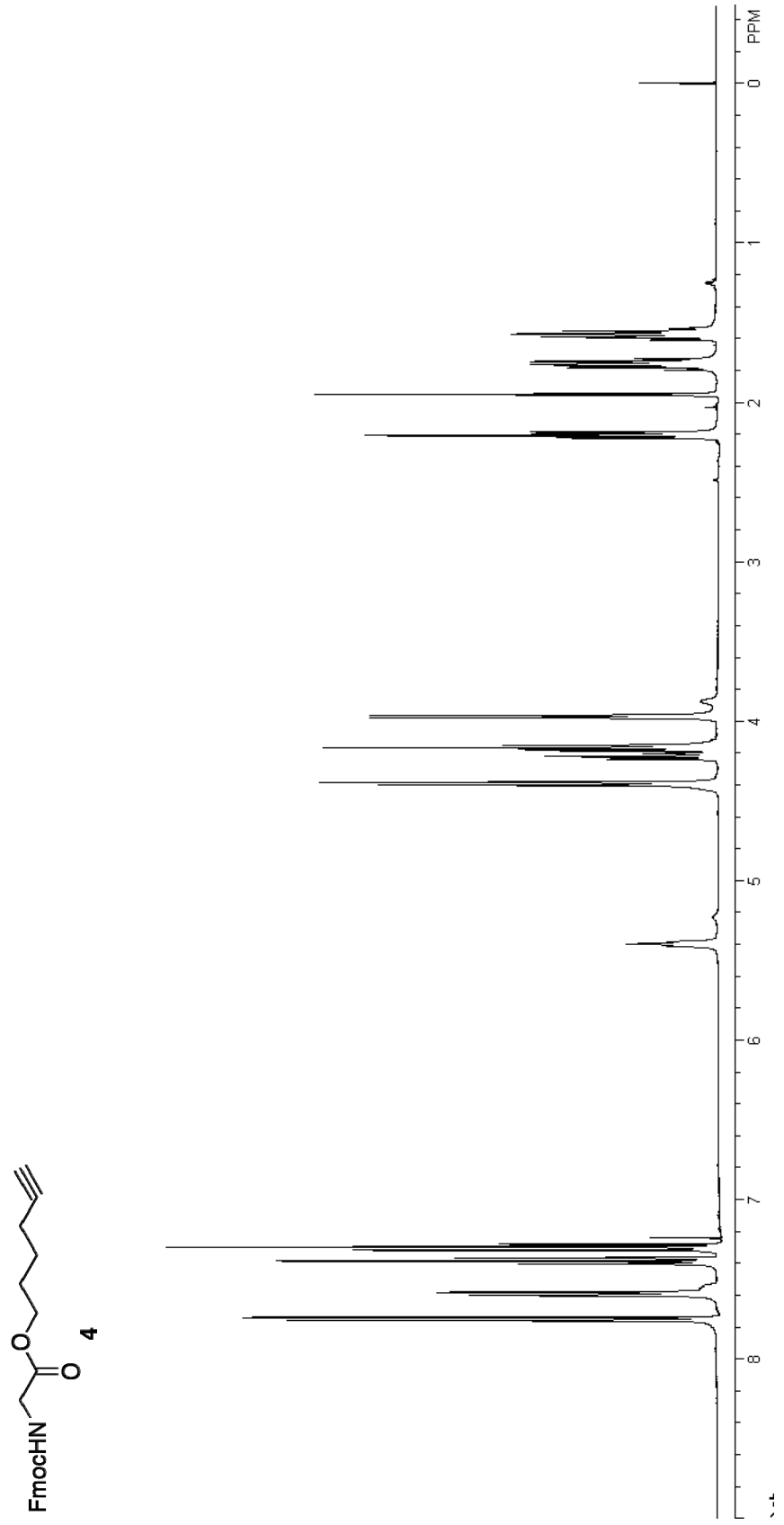
N
N
O
O
1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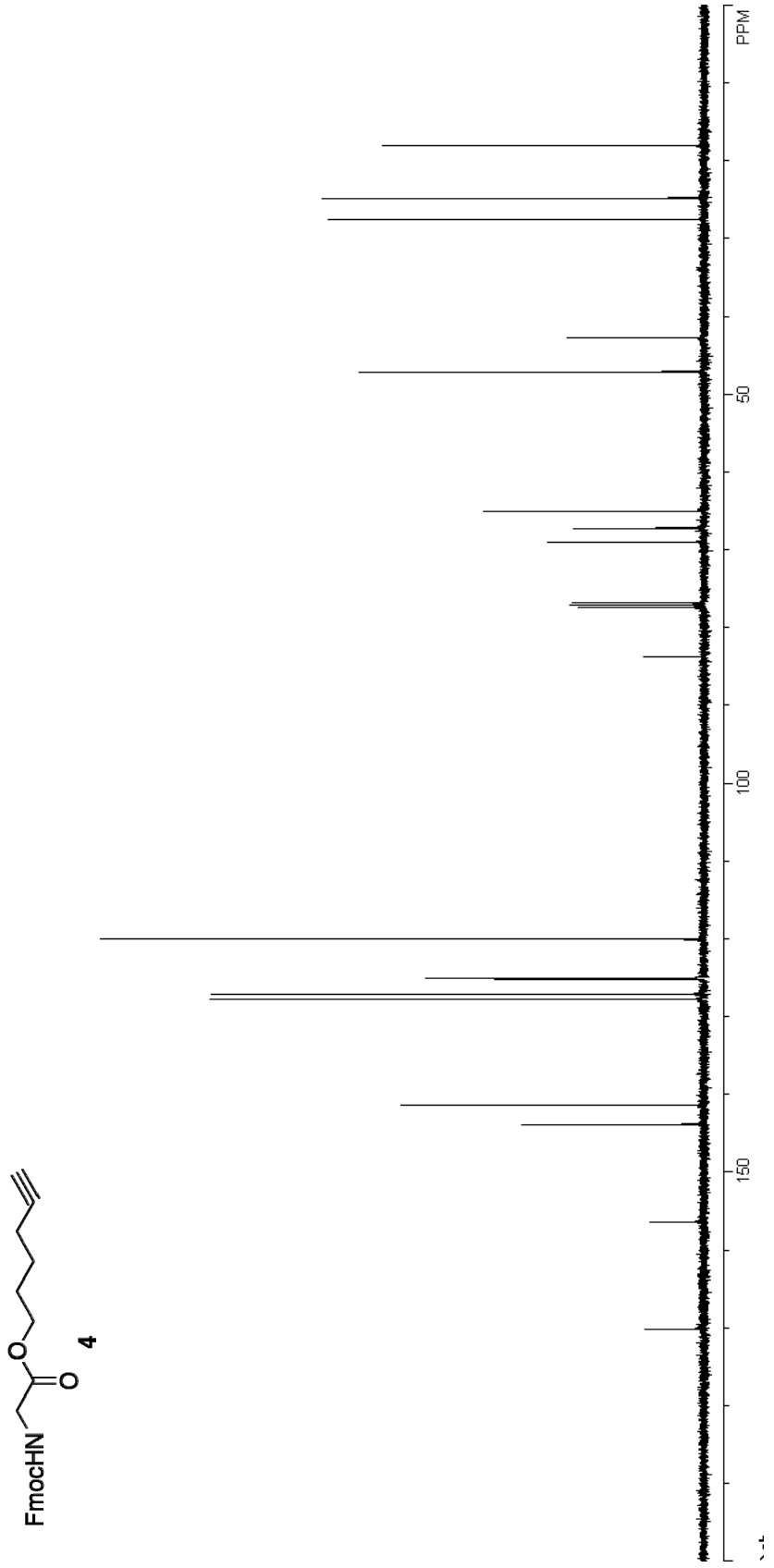
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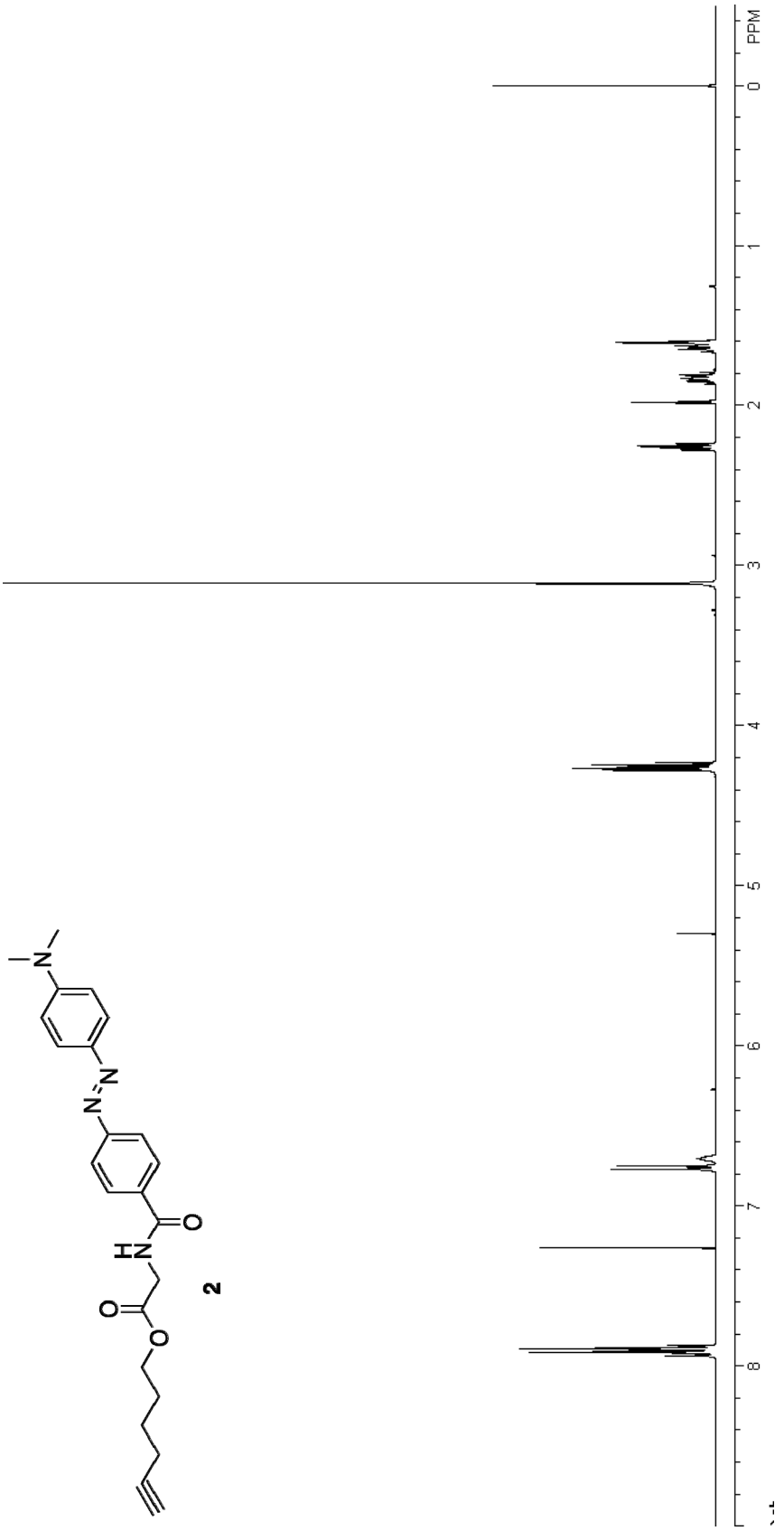

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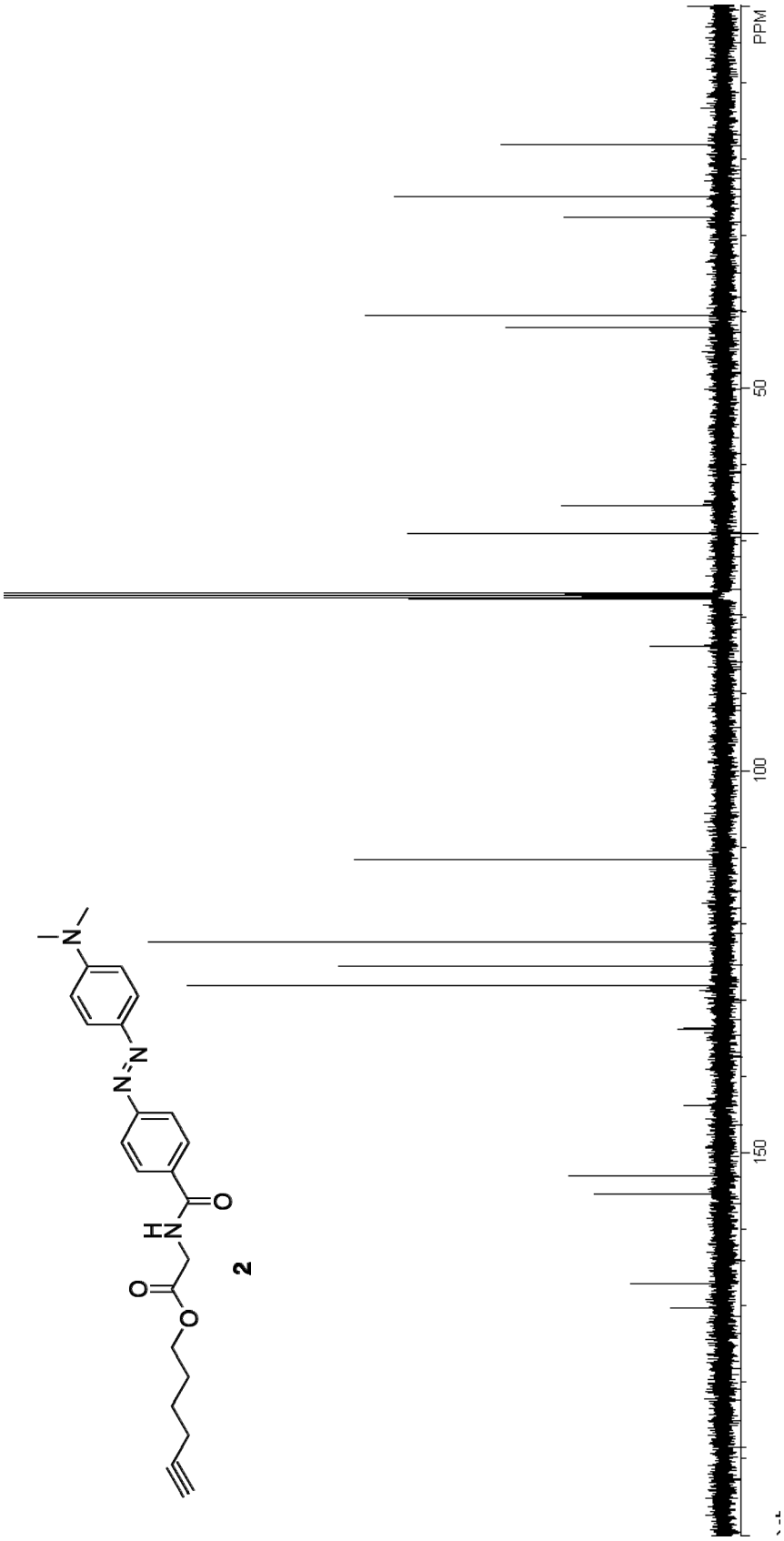
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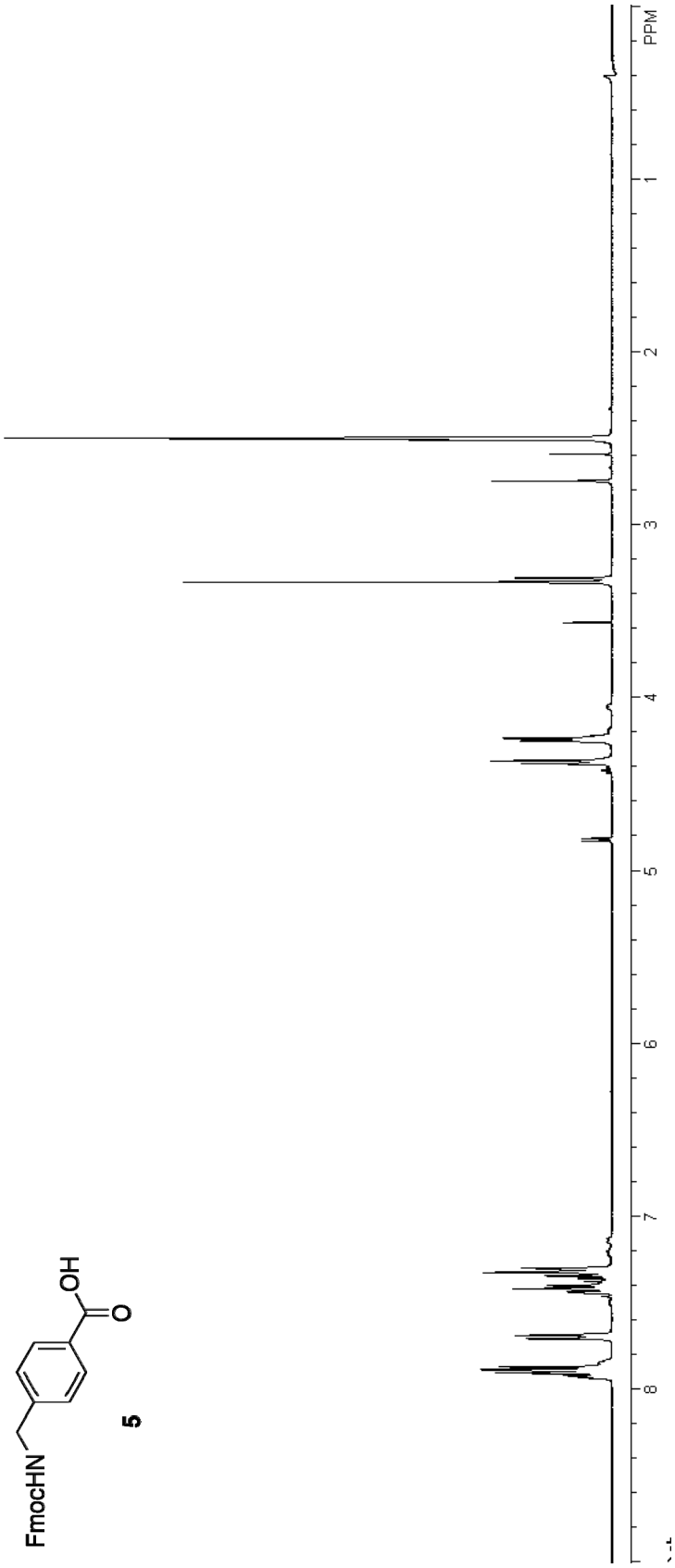
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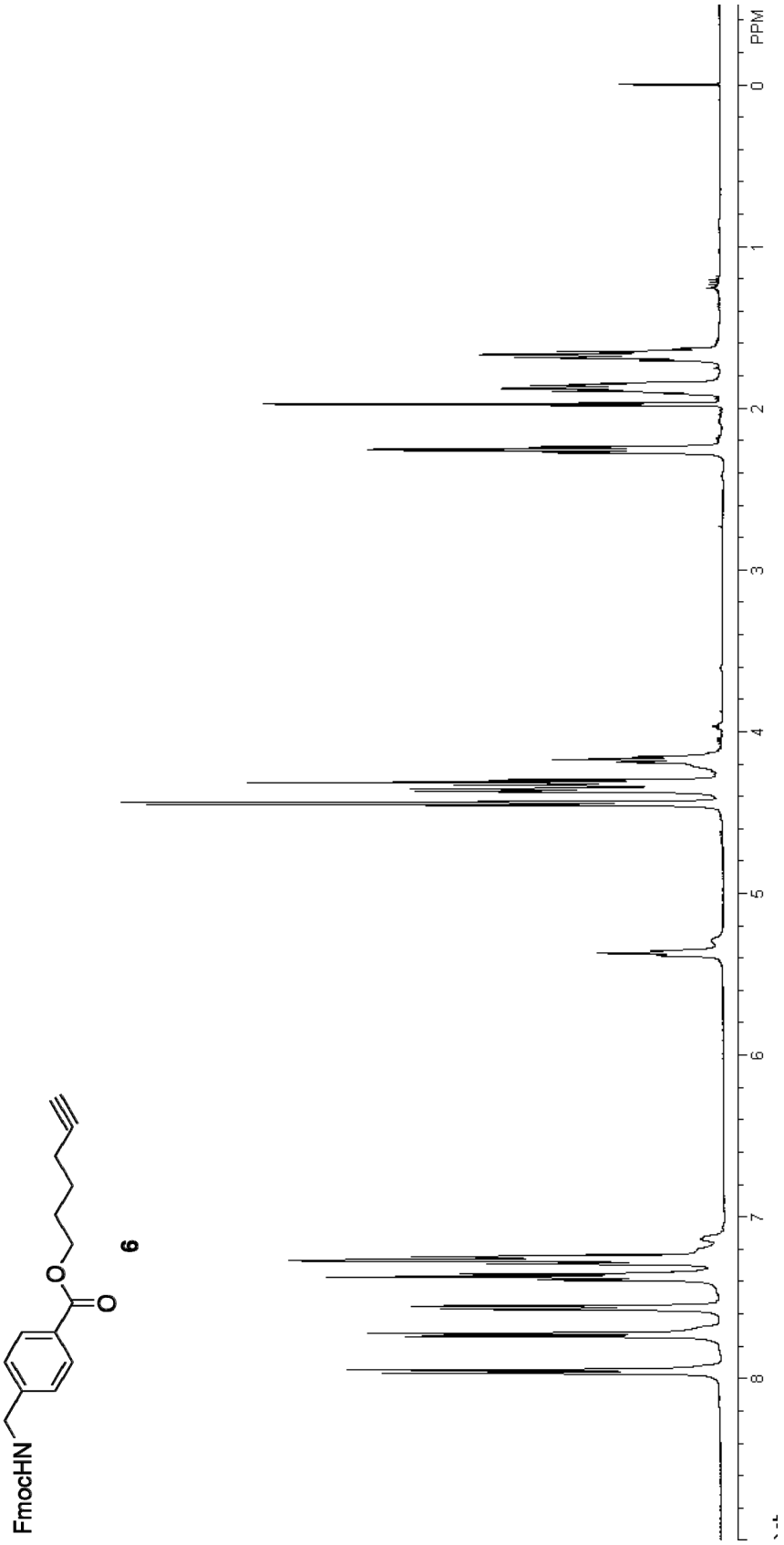

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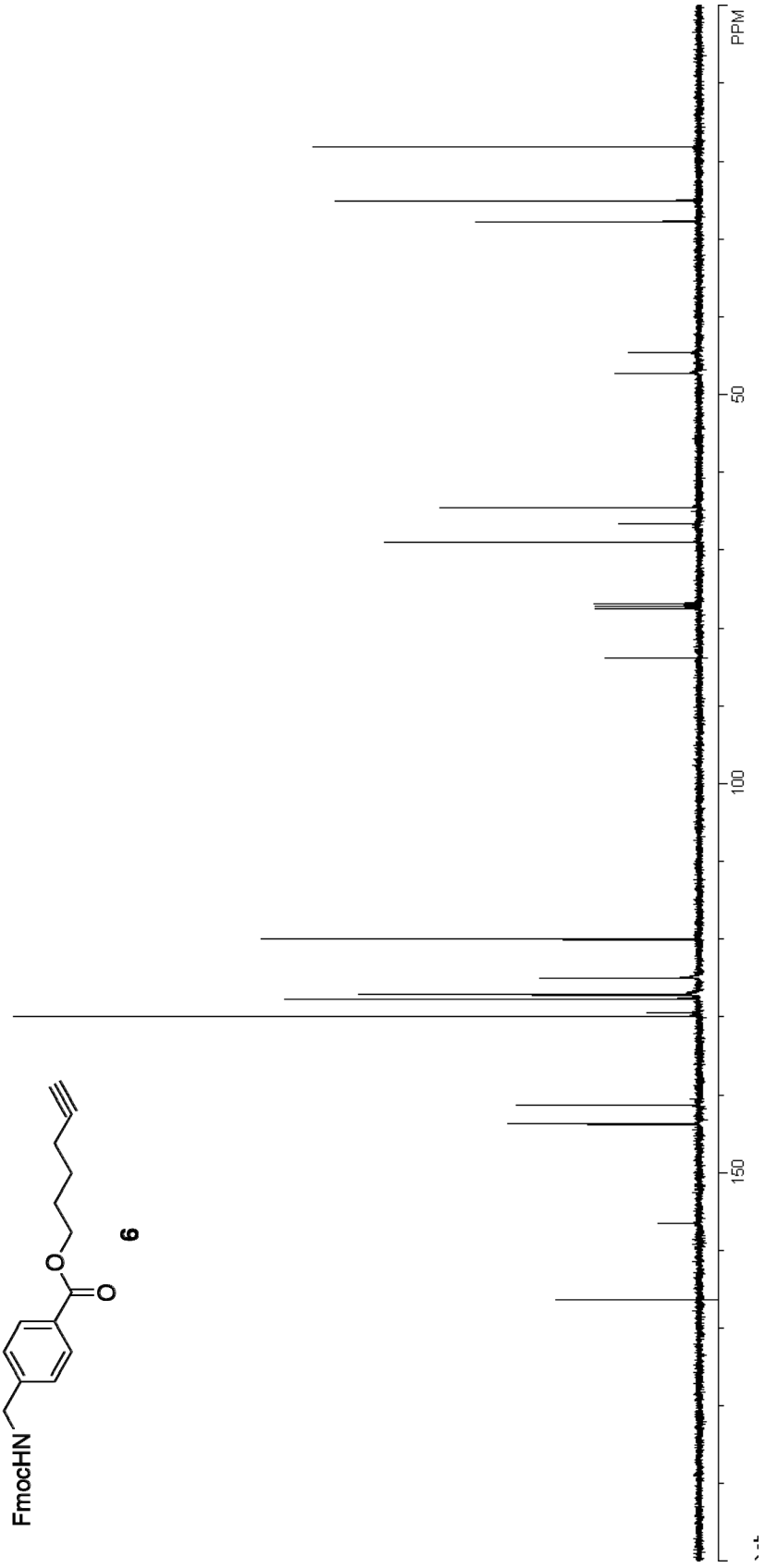
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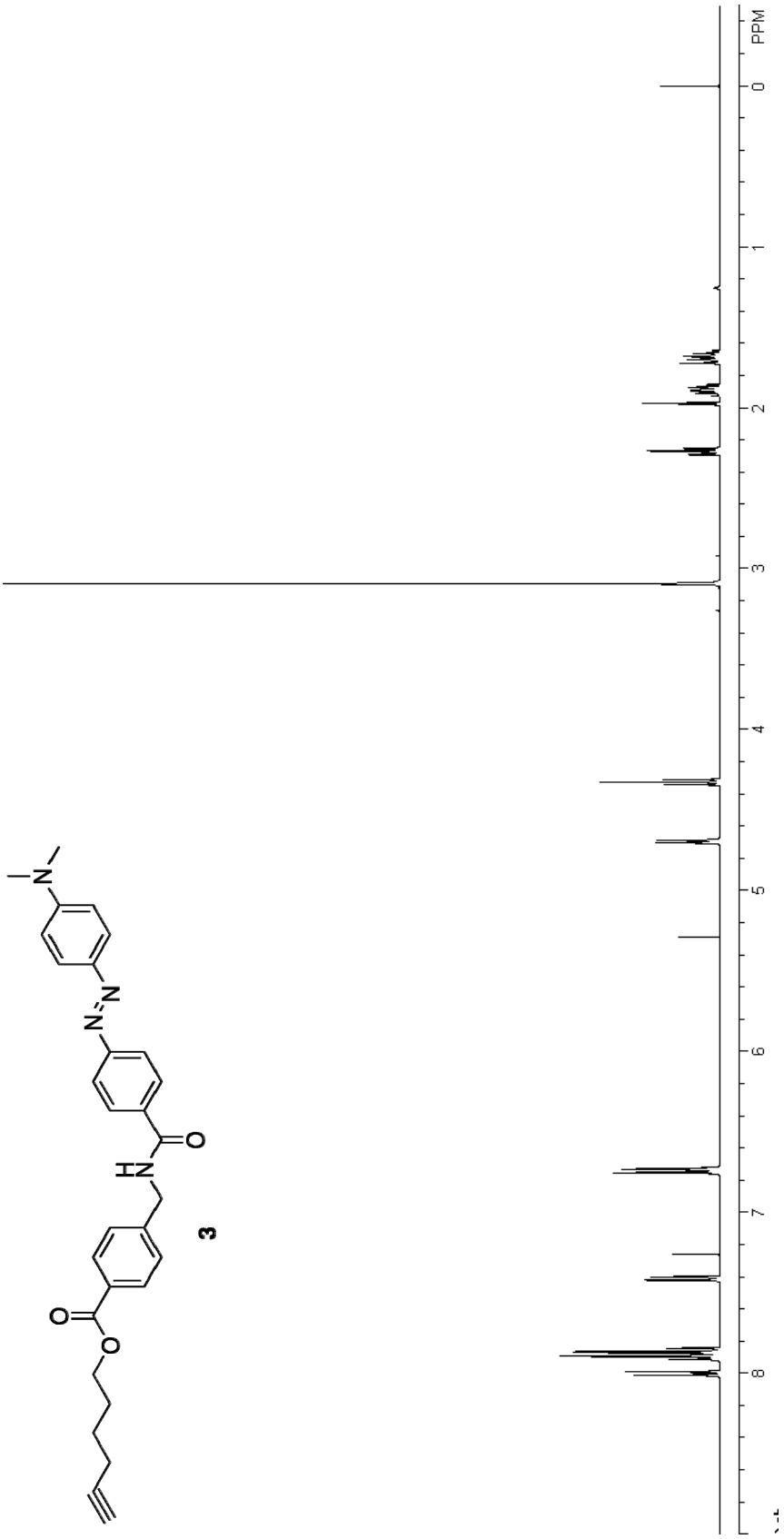

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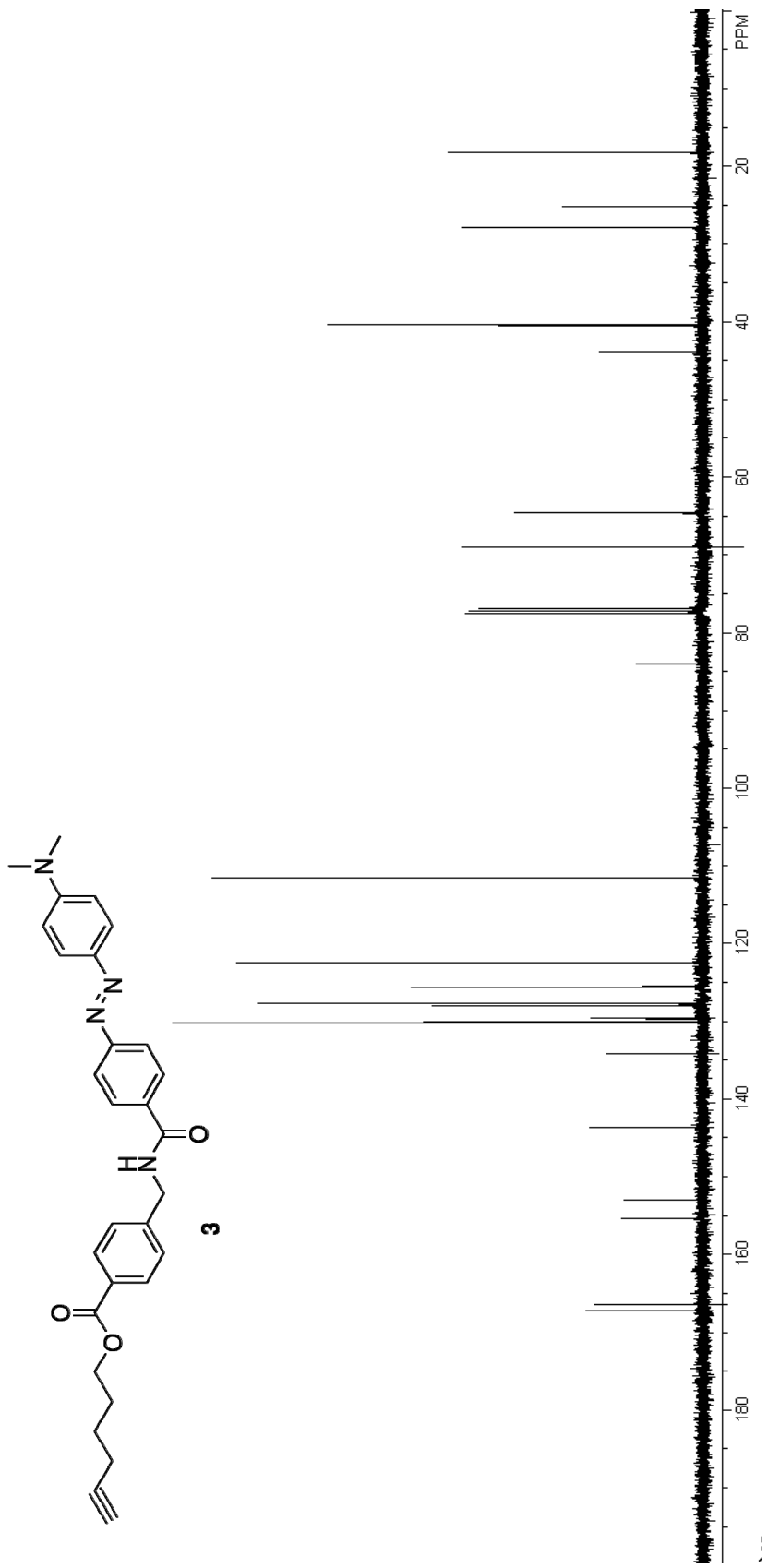
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