Supporting Information

A ratiometric Raman probe for live-cell imaging of hydrogen sulfide in mitochondria by stimulated Raman scattering

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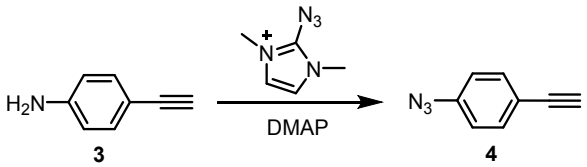
Organic synthesis

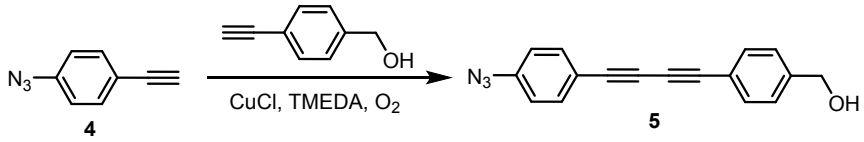
General Information. All reagents and solvents were purchased from Sigma-Aldrich and Fisher Scientific and were used without further purification, unless otherwise stated. Flash chromatography was performed on silica gel (Silicycle, 40-63 μm). TLC was performed on 5 mm E. Merck silica plates (60F-254) and visualized by UV light or potassium permanganate ($KMnO_4$) or ceric ammonium molybdate (CAM) stain. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker 500 (500MHz) or Bruker 400 (400 MHz) Fourier Transform (FT) NMR spectrometers at Columbia University, Chemistry Department. NMR spectra were calibrated using residual undeuterated solvent (^{1}H: δ 7.26 for $CDCl_3$, δ 2.92 for DMF-d_7; ^{13}C: δ 77.16 for $CDCl_3$, δ 162.38 for DMF-d_7). The following abbreviations were used to explain multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad. High resolution mass spectra (HRMS) were recorded on a XEVO G2-XS Waters mass spectrometer equipped with a QTOF detector with multiple inlet and ionization capabilities. UV-Vis absorption spectra were measured on a Tecan infinite 200 using 96-well plates.



3 4

To a solution of 4-ethynylaniline (117 mg, 1.0 mmol) and DMAP (183 mg, 1.5 mmol) in dichloromethane (5 mL) was added ADMP (570 mg, 2.0 mmol) at room temperature. The mixture was heated to 50 °C and stirred for 16 h. After cooling to room temperature, saturated aqueous sodium bicarbonate was added and the mixture was extracted with ether. The organic phase was dried over Na_2SO_4 and the solvent was removed under reduced pressure. The residue was purified by flash column chromatography (hexane/EtOAc = 10/1) to afford 4-ethynylphenyl azide **4** (89 mg, 62%) as a brown oil.

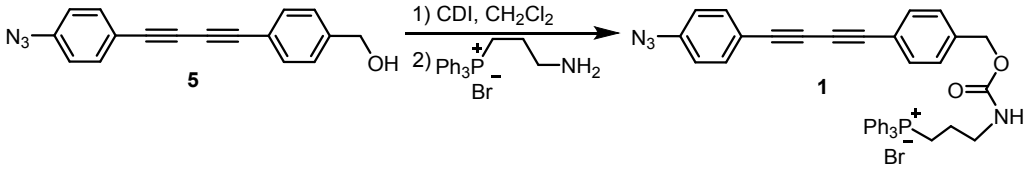
^{1}H NMR (500 MHz, $CDCl_3$) δ7.47 (d, *J* = 8.0 Hz, 2H), 6.97 (d, *J* = 8.0 Hz, 2H), 3.09 (s, 1H);
^{13}C NMR (101 MHz, $CDCl_3$) δ 140.7, 133.7, 119.1, 118.7, 83.0, 77.6.



4 **5**

A solution of CuCl (30 mg, 0.3 mmol) and TMEDA (90 μL, 0.9 mmol) in acetone (2 ml) was bubbled with air for 10 mins at rt, then a mixture of 4-ethynylbenzyl alcohol (66 mg, 0.5 mmol) and 4-ethynylphenyl azide (86 mg, 0.6 mmol) in CH_2Cl_2 (2 ml) were added and continued to stir with air at rt for 2 h. The solvent was evaporated at reduced pressure and the residue was subjected to chromatography to obtain **5** (74 mg, 54%) as a yellow solid.

^{1}H NMR (500 MHz, DMF-d_7) δ 7.71 (d, *J* = 8.0 Hz, 2H), 7.62 (d, *J* = 8.0 Hz, 2H), 7.47 (d, *J* = 8.0 Hz, 2H), 7.22 (d, *J* = 8.0 Hz, 2H), 5.39 (t, *J* = 5.5 Hz, 1H), 4.67 (d, *J* = 5.5 Hz, 2H);
^{13}C NMR (126 MHz, DMF-d_7) δ 145.58, 141.77, 134.60, 132.69, 127.08, 120.07, 119.38, 117.77, 82.52, 81.29, 74.04, 73.28, 63.40.
HRMS (ASAP): calcd for $C_{17}H_{10}N_3^+$ [M-OH]$^+$ 256.0875, found 256.0883.

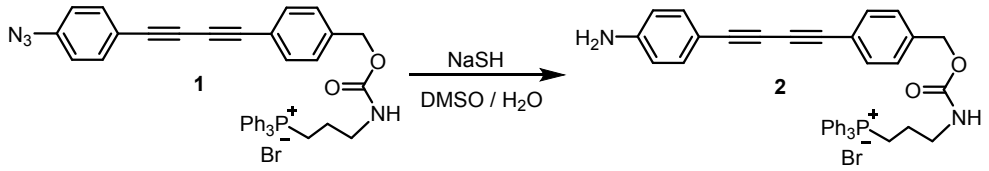


5 **1**

To **5** (10.8 mg, 0.04 mmol) in CH_2Cl_2 (1 ml) was added 1,1'-carbonyldiimidazole (9.7 mg, 0.06 mmol) at rt. After the mixture was stirred at room temperature for 3 h, H_2O was added and the mixture was extracted with CH_2Cl_2. The organic phase was dried over Na_2SO_4 and the solvent was evaporated under reduced pressure. The residue was dissolved in CH_2Cl_2 (1 ml) and (3-aminopropyl) triphenylphosphonium bromide (20 mg, 0.05 mmol) was added and continued to stir at rt for 20 h, then concentrated at reduced pressure and purified by flash chromatography to obtain **1** as a light yellow solid (19 mg, 68%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.80-7.71 (m, 9H), 7.67-7.61 (m, 6H), 7.50 (d, *J* = 8.0 Hz, 2H), 7.42 (d, *J* = 8.0 Hz, 2H), 7.35 (d, *J* = 8.0 Hz, 2H), 6.98 (d, *J* = 8.0 Hz, 2H), 5.06 (s, 2H), 3.80-3.72 (m, 2H), 3.50-3.44 (m, 2H), 1.93-1.77 (m, 2H);
^{13}C NMR (101 MHz, $CDCl_3$) δ 157.1, 141.1, 138.7, 135.3 (d, *J* = 3.0 Hz), 134.1, 133.4 (d, *J* = 10.0 Hz), 132.6, 130.6 (d, *J* = 12.0 Hz), 127.7, 121.0, 119.3, 118.3,

118.1 (d, J = 86.0 Hz), 81.9, 81.0, 74.4, 74.0, 65.7, 40.6 (d, J = 18.0 Hz), 22.9, 20.0 (d, J = 53.0 Hz);
HRMS (ESI): calcd for $C_{39}H_{32}N_4O_2P^+$ [M-Br]$^+$: 619.2263, found: 619.2280.



To **1** (8.4 mg, 0.012 mmol) in DMSO (0.5 ml) was added NaSH (3.4 mg, 0.06 mmol) as a solution in H_2O (0.5 ml) at rt. After the mixture was stirred at room temperature for 3 h, H_2O was added and the mixture was extracted with $CHCl_3$. The organic phase was dried over Na_2SO_4 and the solvent was evaporated under reduced pressure. The residue was purified by column chromatography to afford **2** as a yellow solid (6.6 mg, 82%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.80-7.74 (m, 3H), 7.70-7.61 (m, 12H), 7.39 (d, J = 10.0 Hz, 2H), 7.34 (d, J = 8.5 Hz, 2H), 7.30 (d, J = 8.5 Hz, 2H), 6.58 (d, J = 8.5 Hz, 2H), 5.06 (s, 2H), 3.67-3.60 (m, 2H), 3.47-3.41 (m, 2H), 1.84-1.77 (m, 2H);
^{13}C NMR (126 MHz, $CDCl_3$) δ 157.2, 147.8, 138.5, 135.2 (d, J = 3.6 Hz), 134.2, 133.5 (d, J = 10.0 Hz), 132.5, 130.6 (d, J = 12.6 Hz), 127.8, 121.5, 118.3 (d, J = 85.6 Hz), 114.7, 110.6, 83.0, 80.8, 74.7, 72.1, 65.7, 40.5 (d, J = 17.6 Hz), 22.9, 20.1 (d, J = 51.6 Hz);
LRMS (ESI): calcd for $C_{39}H_{34}N_2O_2P^+$ [M-Br]$^+$: 593.2, found: 593.3.

Stimulated Raman scattering (SRS) microscopy

The laser for SRS microscopy is produced by picoEMERALD (Applied Physics & Electronics, Inc.). A tunable pump beam (720–990 nm, 5–6 ps) and a Stokes beam (1,064 nm, 6 ps) with 80 MHz repetition rate are synchronized. An electro-optic modulator is used to modulate the Stokes beam at 8 MHz. The pump beam is spatially and temporally overlapped with the Stokes beam. They are introduced into an inverted laser-scanning microscope (FV1200, Olympus) and then focused onto the sample by a 25× water objective (XLPlan N, 1.05 N.A., Olympus). The pump and Stokes beams are collected by a high N.A. oil condenser lens (1.4 N.A., Olympus). The Stokes beam is blocked by a bandpass filter (890/220 CARS, Chroma Technology) and the pump beam is measured by a large-area (10 mm × 10 mm) Si photodiode (FDS1010, Thorlabs). The output current terminated with 50 Ω is filtered by an 8 MHz electronic bandpass filter (KR 2724, KR electronics). The stimulated Raman loss signal is demodulated by a lock-in amplifier (SR844, Stanford Research Systems). The output of the lock-in amplifier is sent to an analogue interface box (FV10-ANALOG, Olympus) of the microscope and images are acquired by Fluoview software (Olympus). We use 100 μs pixel dwell time for a 512×512 pixel field of

view and set 30 μs time constant for the lock-in amplifier to acquire the SRS images. The measured pump and Stokes powers on sample are 20 mW and 80 mW respectively. Fluorescence images were acquired by an Olympus FV1200 confocal microscope and the excitation wavelength is 635 nm. All images are processed by ImageJ.

Cell culture and imaging

Both HeLa and COS-7 cells were purchased from ATCC and cultured in incubator at 37 °C under the atmosphere with 5% CO_2. Culture media contain 10% (v/v) FBS and 1% (v/v) P&S of DMEM. Cells were seeded and cultured on a glass coverslip for 24 h and incubated with corresponding probe. After incubation, the cell samples were washed with PBS and then the SRS imaging and fluorescence imaging were performed.

Co-localization imaging of living cells. Cells were incubated with 10 μM **1** or **2** for 0.5 h. Then 0.2 μM Mito Tracker Deep Red was added and incubated for another 0.5 h at 37 ℃. The SRS and fluorescence images (excitation wavelength at 635 nm) were captured.

Ratiometric imaging of probe 1 in living cells. Cells were incubated with 10 μM **1** in culture media for 0.5 h at 37 ℃. Subsequently, the cells were imaged at 2212 cm^{-1}, 2228 cm^{-1}, 2000 cm^{-1}, and 2941 cm^{-1} using SRS microscopy.

Ratiometric imaging of 1 toward H_2S in living cells. Cells were incubated with 10 μM **1** in culture media for 0.5 h at 37 ℃. Then the culture media was replaced by a new DMEM medium with 200 μM NaSH and incubated for 1h. Subsequently, the cells were imaged at 2212 cm^{-1}, 2228 cm^{-1}, 2000 cm^{-1}, and 2941 cm^{-1} using SRS microscopy.

Reaction kinetics responses to H_2S. A 50 μL 2-yne Mito N_3 **1** (0.5 mM) was added to degassed PBS (450 μL) and 5 μL NaSH solution (50 mM in degassed PBS) was added. The mixtures in PBS were stirred at room temperature for 0, 15, 30, 60, 90 and 120 min after addition of NaSH. The reaction mixtures were then extracted with $CHCl_3$ and the solvents were concentrated, then the residues were dissolved in DMSO for SRS measurement.

Cytotoxicity assays. HeLa cells were incubated with 10 μM **1** for 3 h at 37 ℃. The Live/Dead viability/cytotoxicity kit for mammalian cells (Invitrogen, L3224) were used for cell viability assays by incubating cells with 2 μM calcein AM and 4 μM EthD-1 working solution for 20 min at 37 °C.

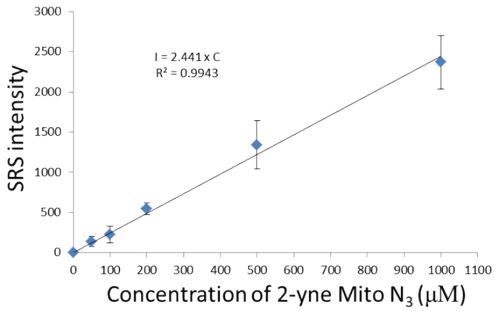


Fig. S1 Linear relationship between measured SRS intensity at 2223 cm^{-1} and 2-yne Mito N_3 **1** in DMSO solutions at different concentrations (50, 100, 200, 500 and 1000 μM) with a detection limit (S/N=1) of 15 μM based on the system noise.

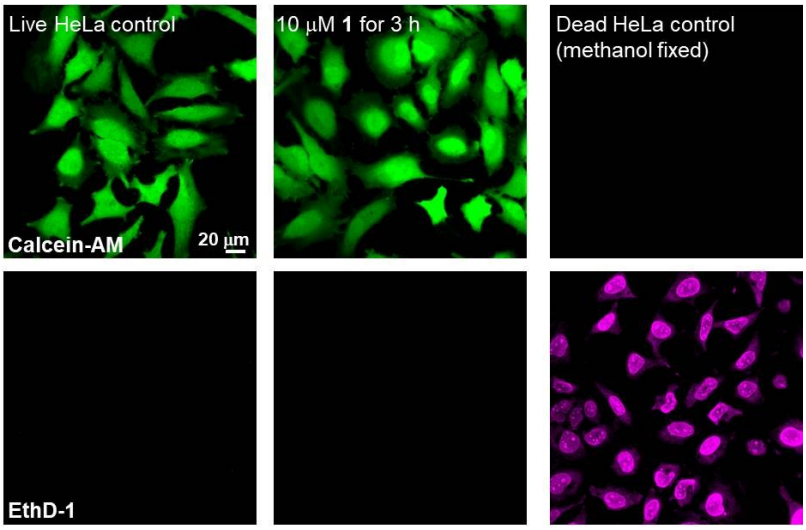


Fig. S2 Minimal cytotoxicity of Raman probe **1** in HeLa cells. Live and dead HeLa cells are stained with Calcein-AM (green, live-cell marker) and EthD-1 (magenta, dead-cell marker).

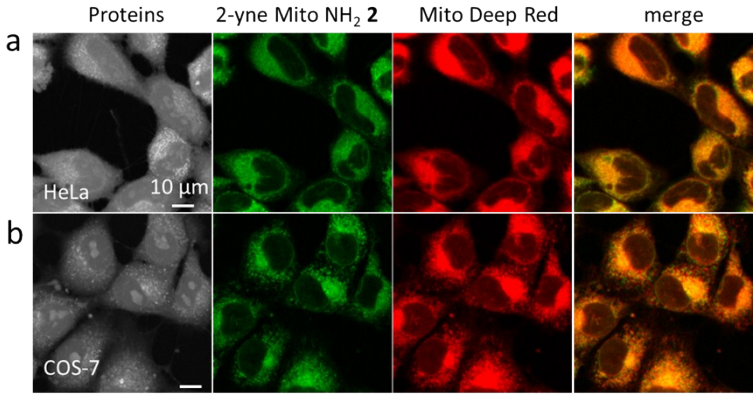
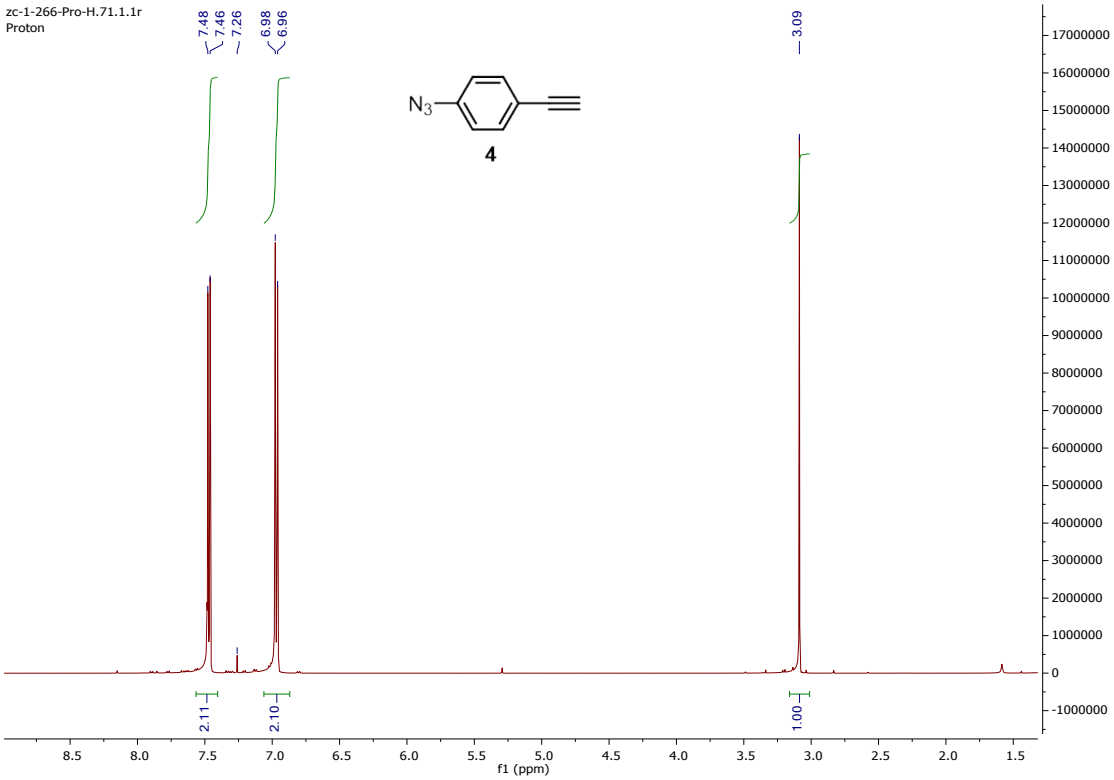
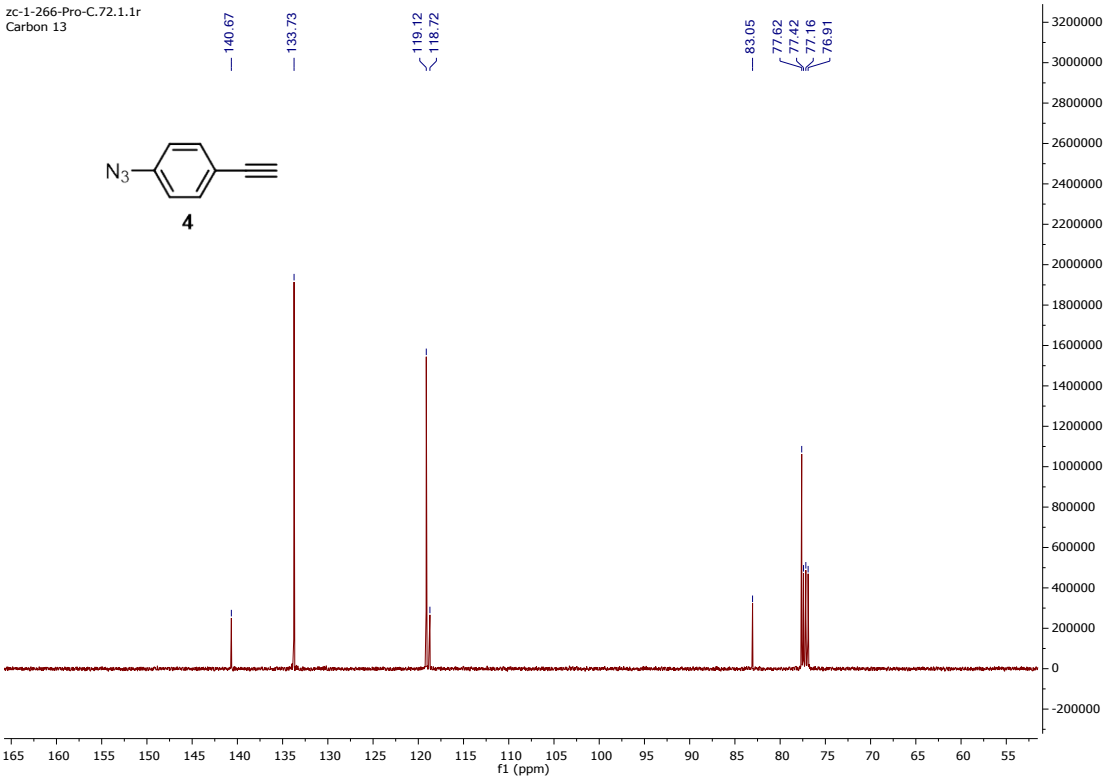
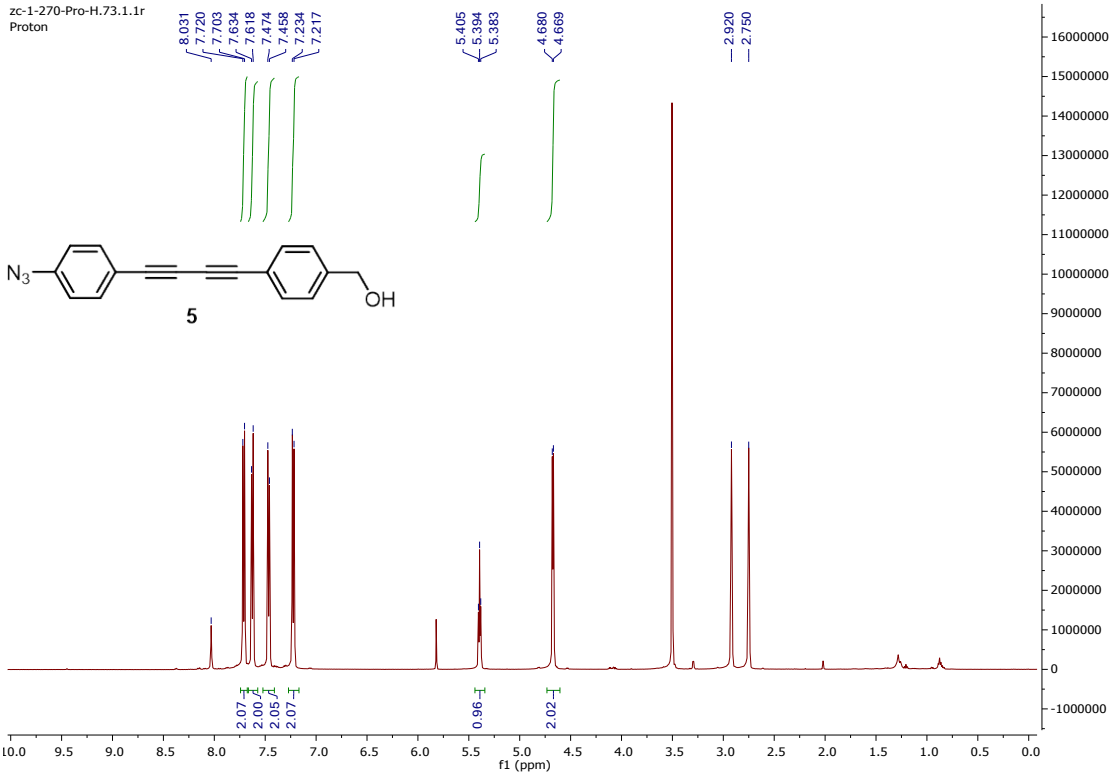


Fig. S3 Co-localization imaging of **2** in living cells. (a) HeLa or (b) COS-7 cells were incubated with 10 μM **2** for 1 h and 0.2 μM Mito Tracker Deep Red for 0.5 h at 37 ℃. Scale bar: 10 μm.

^{1}H NMR and ^{13}C NMR spectra of intermediates and Raman probes



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7.474
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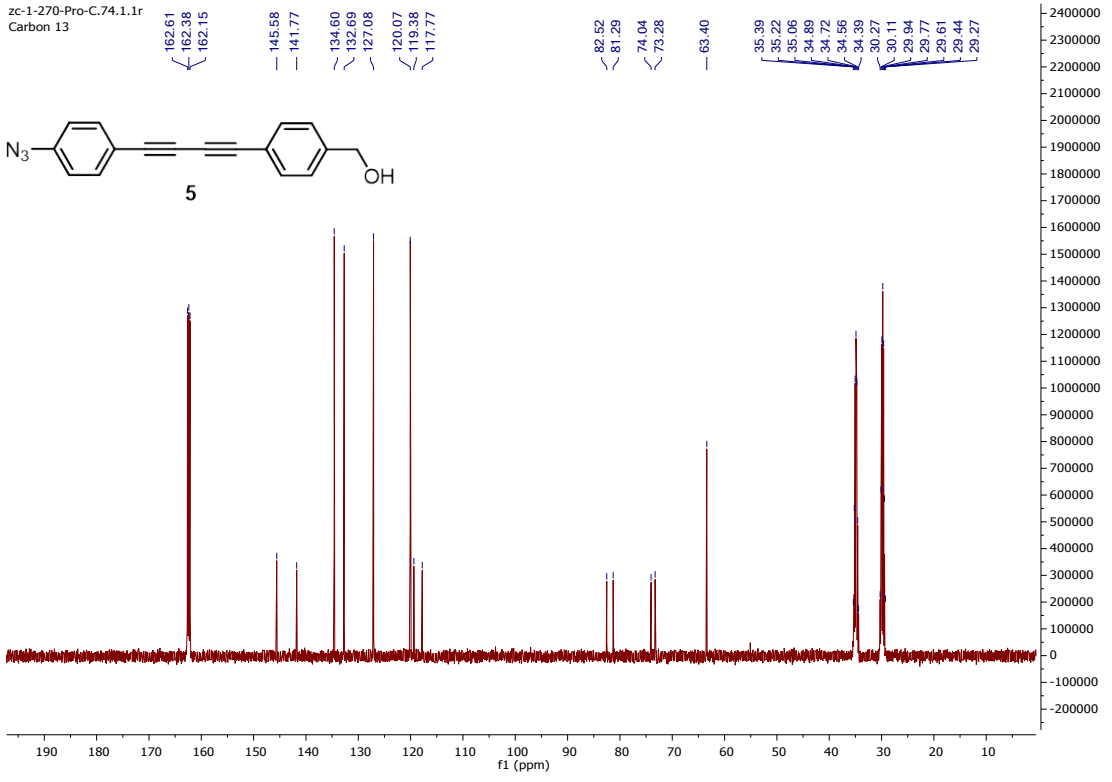
5.405
5.394
5.383
4.680
4.669
2.920
2.750
N3
OH
5
2.07
2.00
2.05
2.07
0.96
2.02
f1 (ppm)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Carbon 13
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162.38
162.15
145.58
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134.60
132.69
127.08
120.07
119.38
117.77
82.52
81.29
74.04
73.28
63.40
35.39
35.22
35.06
34.89
34.72
34.56
34.39
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30.11
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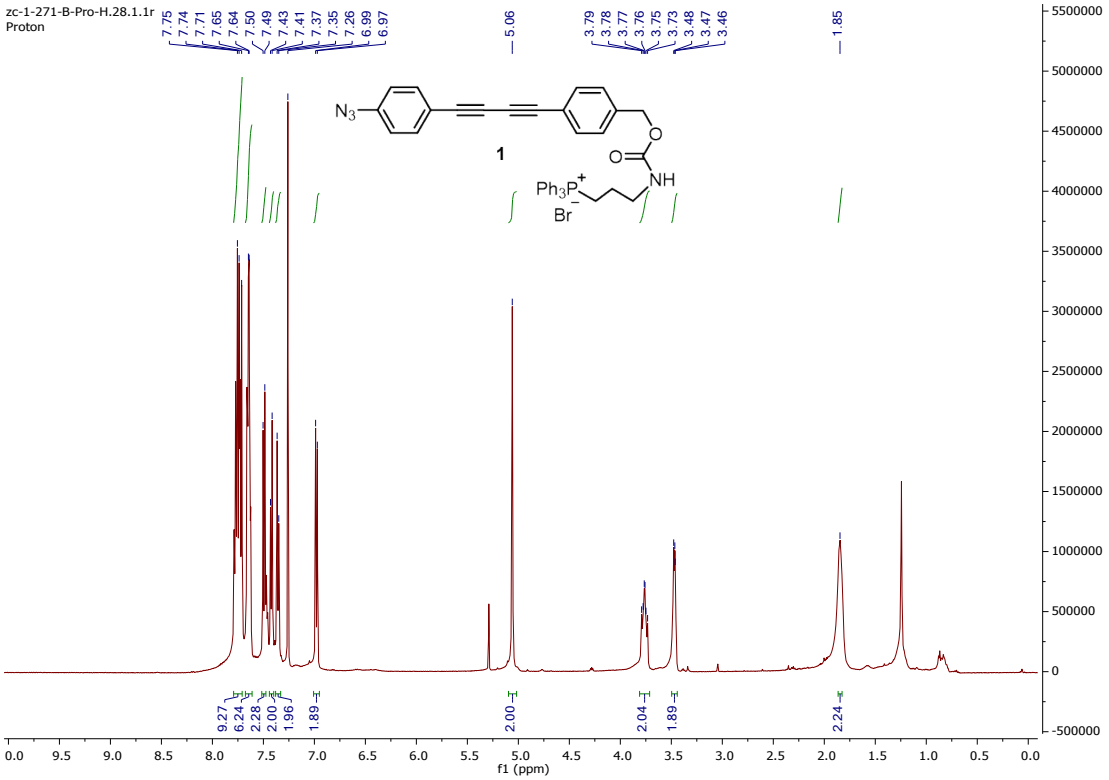
29.61
29.44
29.27
N3
OH
5
f1 (ppm)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7.65
7.64
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7.37
7.35
7.26
6.99
6.97
5.06
3.79
3.78
3.77
3.76
3.75
3.73
3.48
3.47
3.46
1.85
N3
1
O
O
NH
Ph3P+
Br−
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2.28
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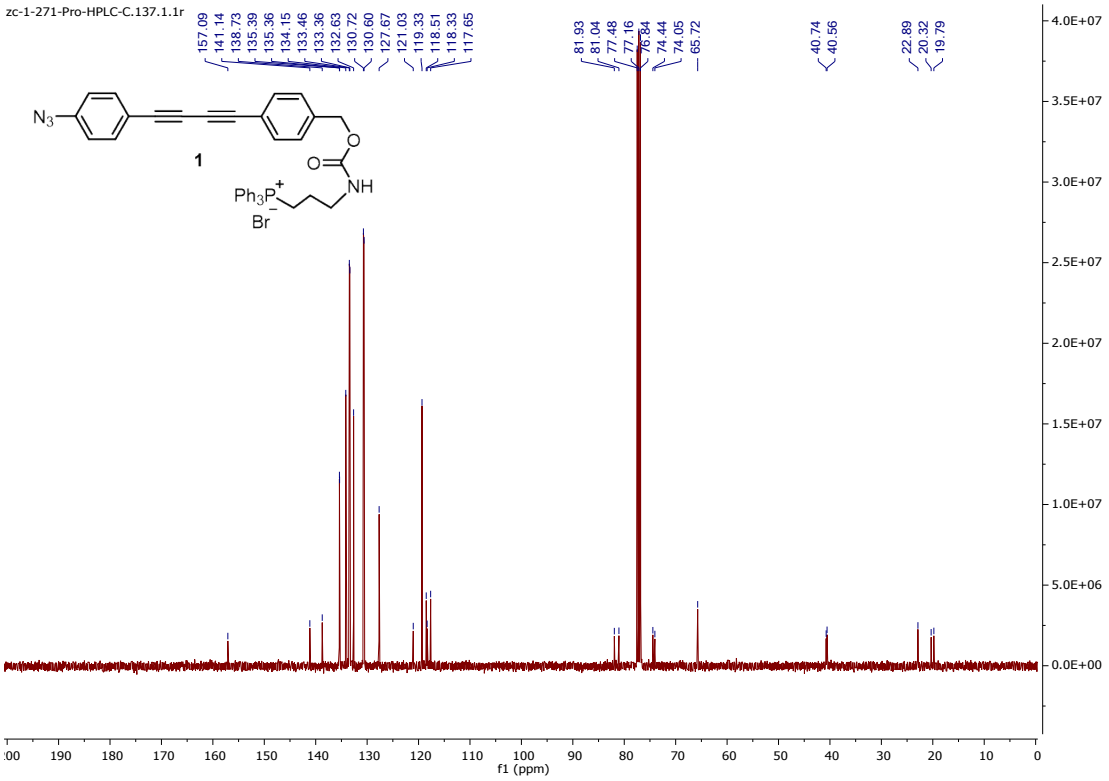
1.96
1.89
2.00
2.04
1.89
2.24
f1 (ppm)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130.60
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121.03
119.33
118.51
118.33
117.65
81.93
81.04
77.48
77.16
76.84
74.44
74.05
65.72
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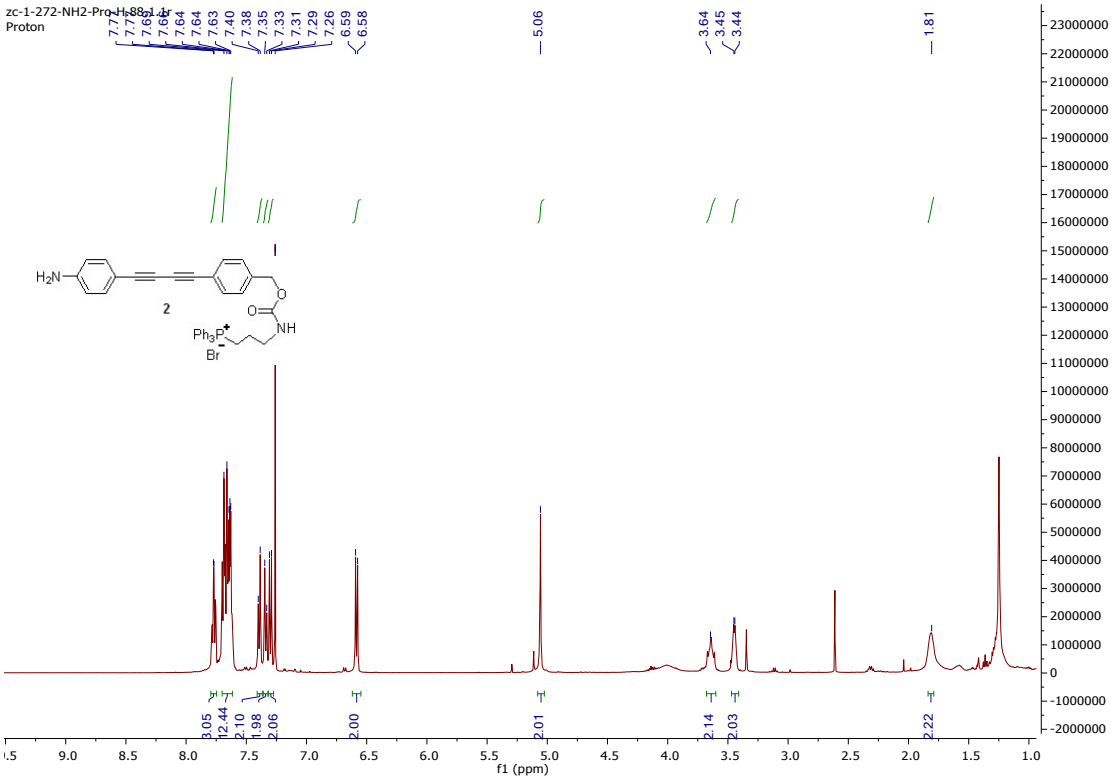
NH
Ph3P+
Br−
f1 (ppm)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7.63
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3.45
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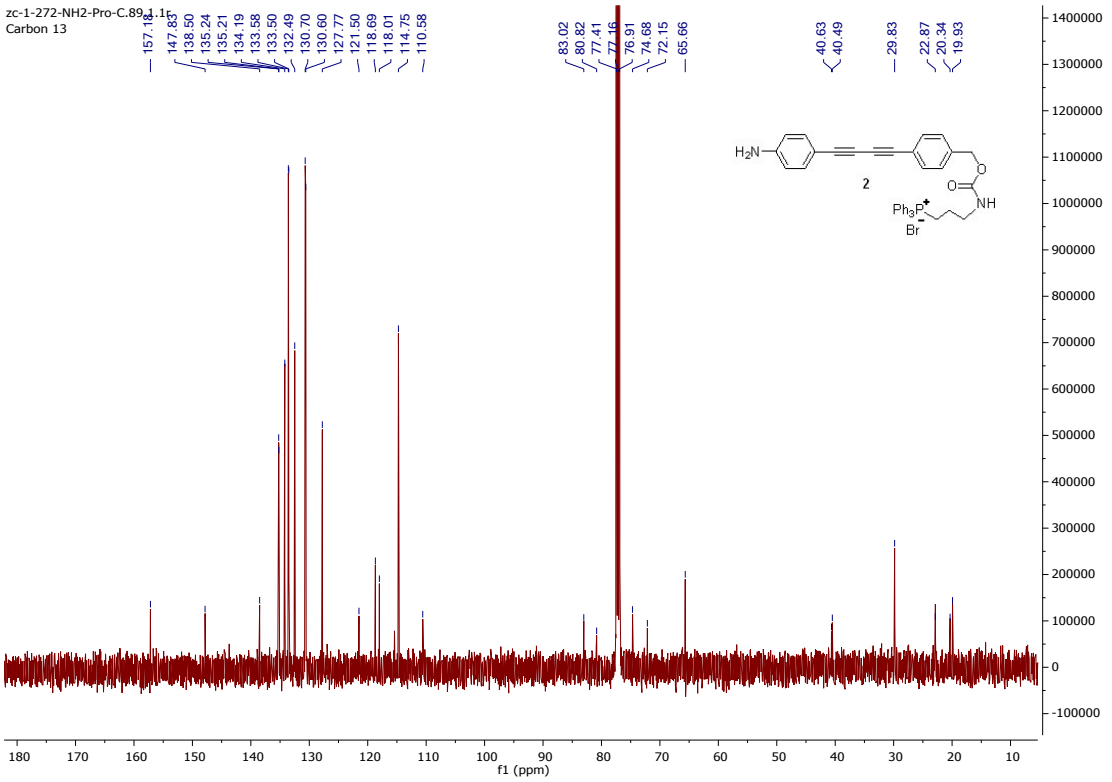
2
f1 (ppm)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130.70
130.60
127.77
121.50
118.69
118.01
114.75
110.58
83.02
80.82
77.41
77.16
76.91
74.68
72.15
65.66
40.63
40.49
29.83
22.87
20.34
19.93
2
f1 (ppm)