Supporting Information

Anaerobic Conditions to Reduce Oxidation of Proteins and to Accelerate Copper-catalyzed "Click" Reaction with a Water-soluble Bis(triazole) Ligand

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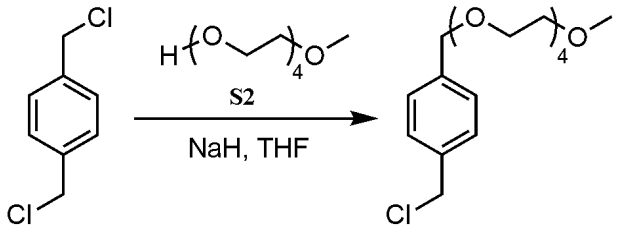
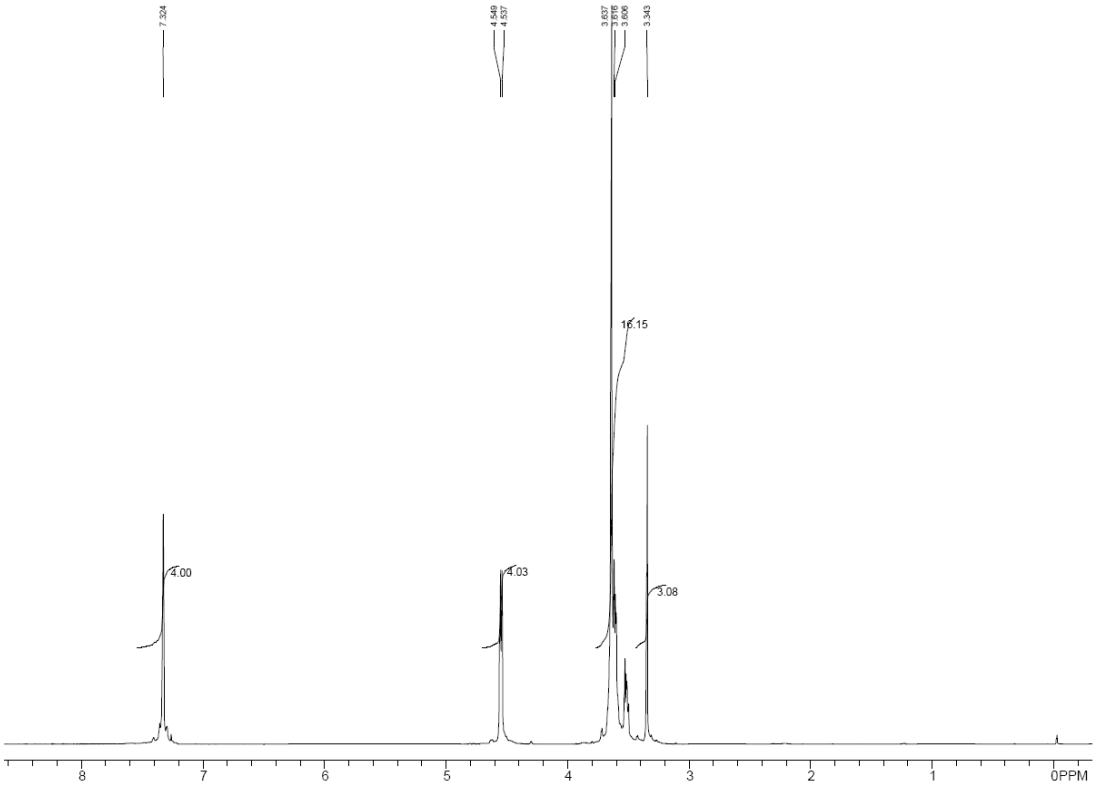
A: Synthesis

General: Air sensitive reactions were performed under a nitrogen atmosphere using Schlenk technique. 1,4-Bis(chloromethyl)benzene (**S1**), tetra(ethylene glycol) monomethyl ether (**S2**), tripropargyl amine (**7**), dipropargyl amine (**S5**), propargyl amine (**S6**) and 4-bromo-1,8-naphthalic anhydride (**S7**) were purchased from Sigma-Aldrich (St. Louis, MO), mono azido tetra(ethylene glycol) (**8**) and mono amino tetra(ethylene glycol) (**S8**) were purchased from Quanta BioDesign (Powell, Ohio), and trimethylsilylacetylene (**S10**) was purchased from 3B Scientific Corporation (Libertyville, IL). All ^{1}H- and ^{13}C-NMR spectra were recorded in GE QE-300 in $CDCl_3$ (Cambridge Isotope Laboratories Inc.) using residual $CHCl_3$ as an internal standard. Mass spectroscopy (MS) measurements were carried out using electrospray ionization (ESI) technique on Deca XP Plus (Thermo Finnigan).



S1 **S3**

To a solution of tetra(ethylene glycol) monomethyl ether (**S2**, 2.00 g, 9.61 mmol) in THF (15 mL) at room temperature was added NaH (276 mg, 11.5 mmol), and the reaction mixture was stirred for 30 min, followed by addition of 1,4-bis(chloromethyl)benzene (**S1**, 2.01 g, 11.5 mmol). The reaction mixture was stirred at room temperature overnight. Solvent was evaporated and the residue was dissolved in CH_2Cl_2 and washed with water. The organic phase was dried with Na_2SO_4 and concentrated under reduced pressure. The residue was purified by flash chromatography (ethyl acetate) to give **S3** (1.82 g, 5.47 mmol, 57%) as a colorless liquid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.26-7.40 (m, 4 H), 4.55 (s, 4 H), 3.75-3.50 (m, 16 H), 3.34 (s, 3 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 139.2, 137.3, 129.2, 128.5, 73.3, 72.5, 71.2, 71.1, 71.0, 70.0, 59.5, 46.5; MS (ESI) *m*/*z* calcd for $C_{22}H_{35}N_6O_{11}$: 346.1; found: 369.2. ($[M + Na]^+$).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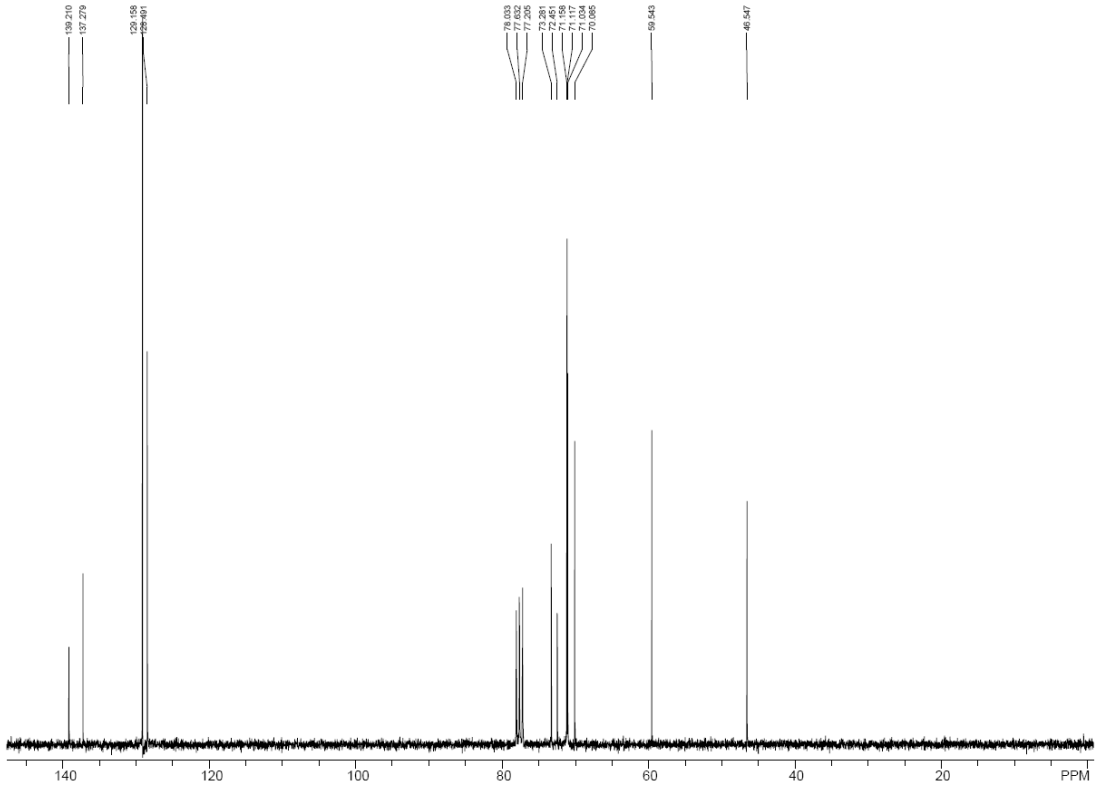
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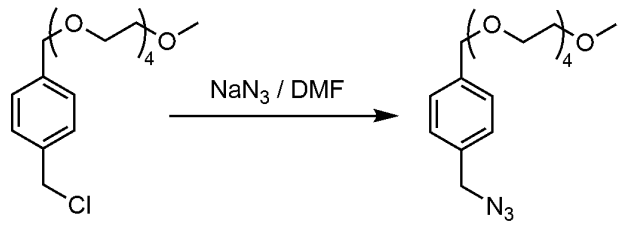
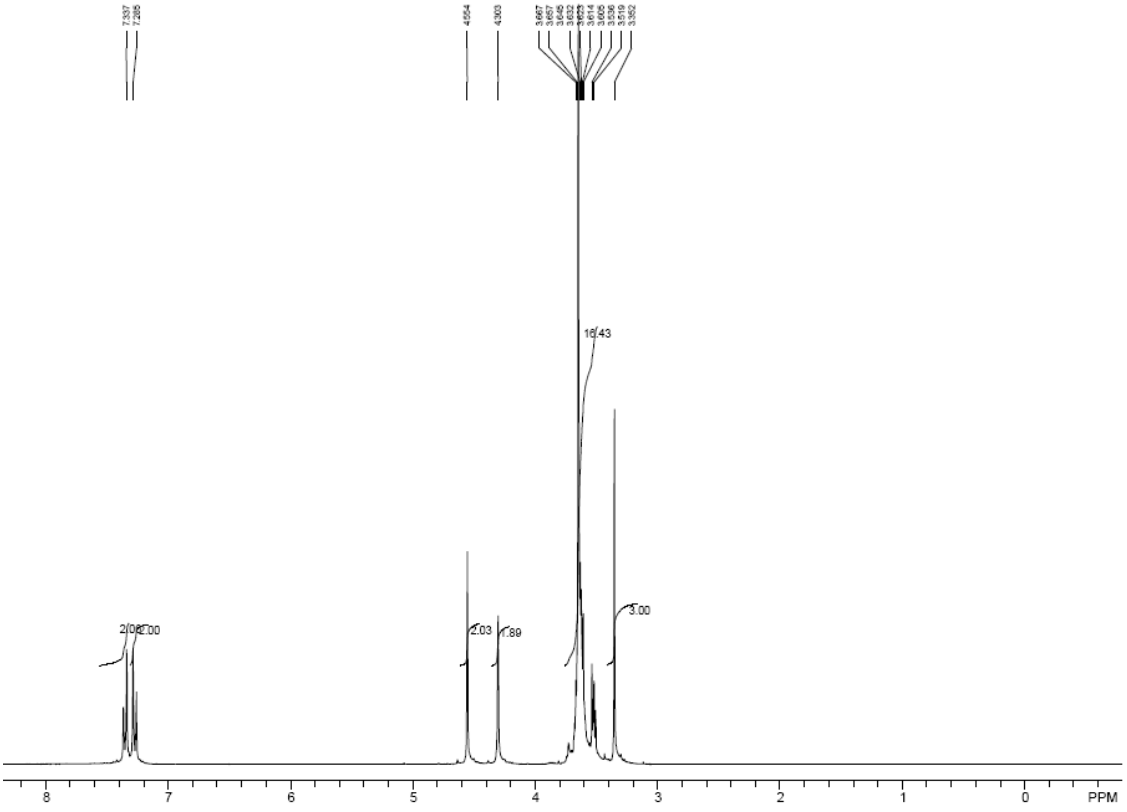
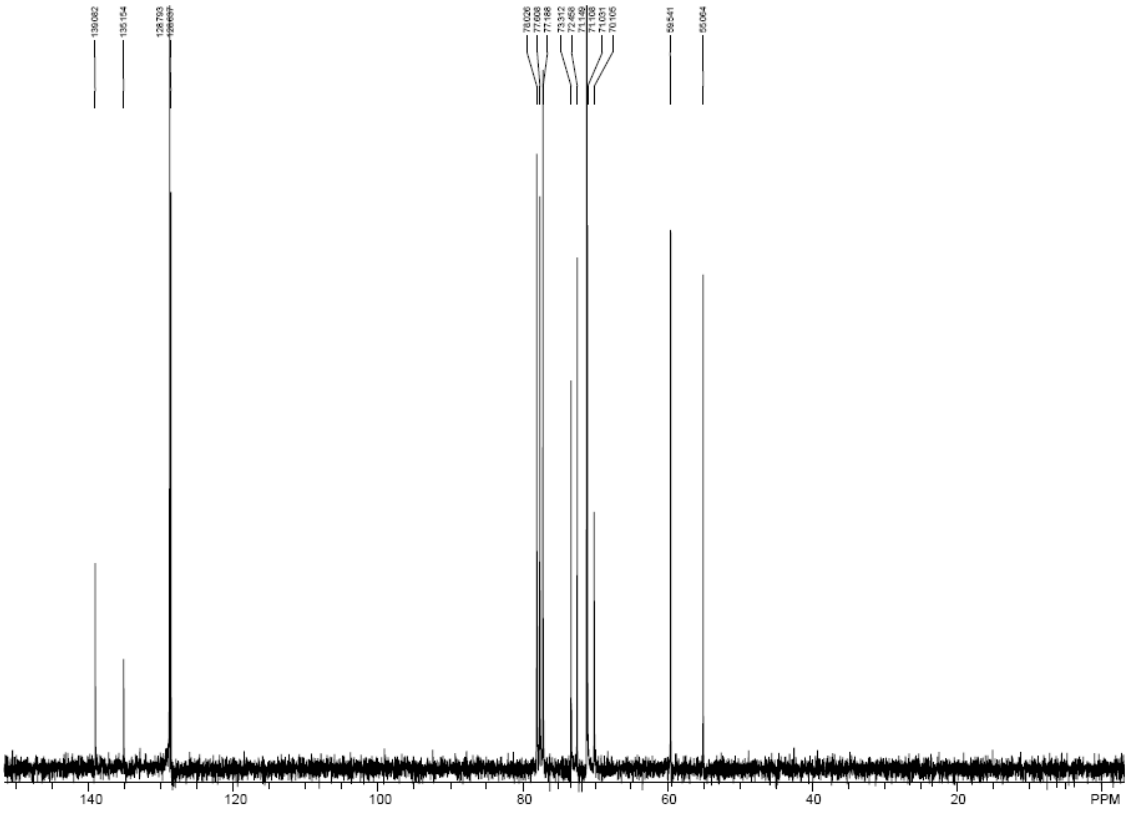
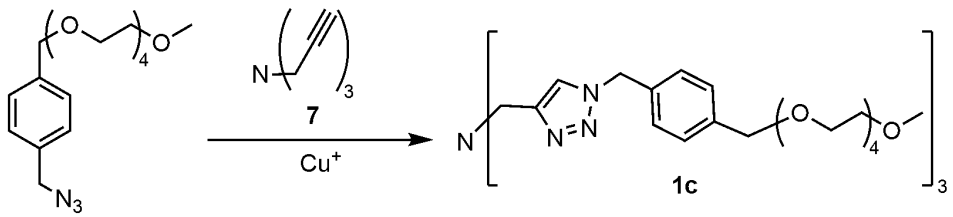


S3 **S4**

To a solution of **S3** (1.50 g, 4.33 mmol) in DMF (10 mL) was added NaN_3 (337 mg, 5.20 mmol), and the reaction mixture was stirred overnight at 110 ˚C. After the solvent was evaporated, the residue was suspended in ethyl acetate, and the mixture was and filtered. The filtrate was concentrated under reduced pressure. The residue was purified by flash chromatography (ethyl acetate) to give **S4** (1.37 g, 3.89 mmol, 90%) as a colorless liquid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.33 (d, 2 H, J = 8.0 Hz), 7.28 (d, 2 H, J = 8.2 Hz), 4.55 (s, 2 H), 4.30 (s, 2 H), 3.75-3.45 (m, 16 H), 3.36 (s, 3 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 139.0, 135.1, 128.7, 128.2, 73.3, 72.5, 71.5, 71.0, 70.0, 59.5, 55.0; MS (ESI) *m*/*z* calcd for $C_{17}H_{27}N_3O_5$: 352.2; found: 375.2 ([M + Na]$^+$).

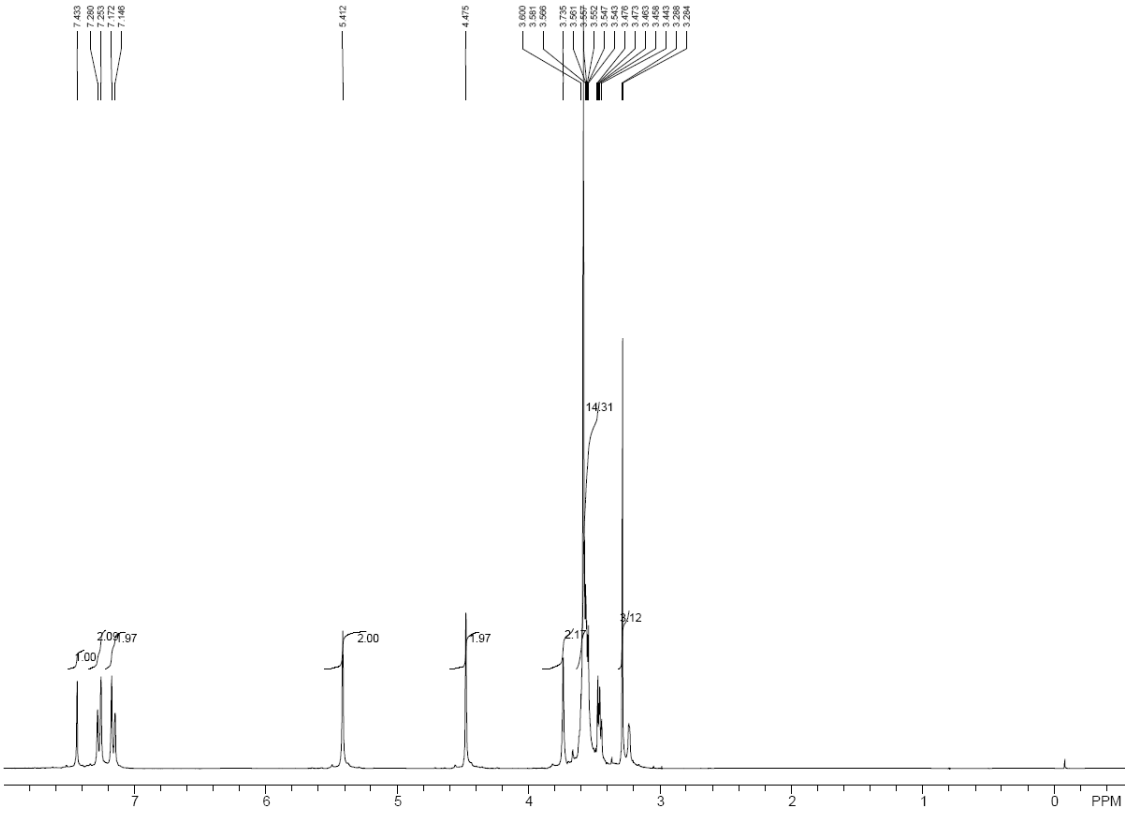






S4

Tripropargylamine (**7**, 82.4 mg, 0.628 mmol) in CH_3CN (1 mL) was treated sequentially with the azide **S4** (1.00 g, 2.83 mmol), 2,6-lutidine (303 mg, 2.83 mmol), and $Cu(MeCN)_4PF_6$ (9.10 mg, 0.020 mmol). After the mixture was stirred at room temperature for 3 days, the solvent was evaporated, and the residue purified by flash chromatography to give **1c** (0.560 g, 0.470 mmol, 74%) as a light brown viscous liquid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.43 (s, 3 H), 7.27 (d, 6 H, J = 7.8 Hz), 7.15 (d, 6 H, J = 8.1 Hz), 5.41 (s, 6 H), 4.47 (s, 6 H), 3.74-3.42 (m, 48 H), 3.28 (s, 9 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 145.2, 139.5, 134.5, 128.7, 128.6, 123.5, 74.3, 73.1, 72.4, 71.0, 70.9, 70.1, 59.5, 54.3; MS (ESI) *m/z* calcd for $C_{60}H_{90}N_{10}O_{15}$: 1190.6; found: 1213.6 ([M + Na]$^+$).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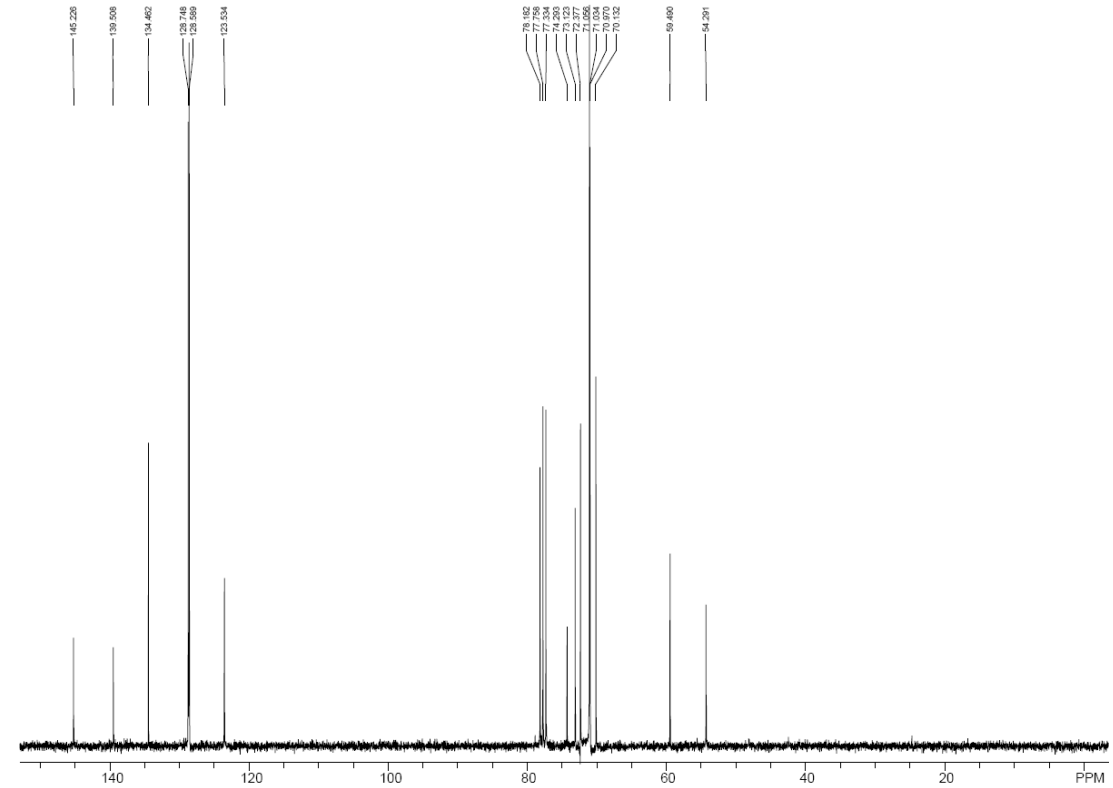
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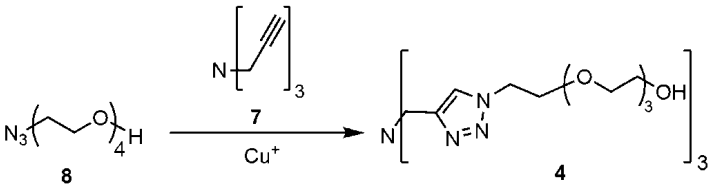
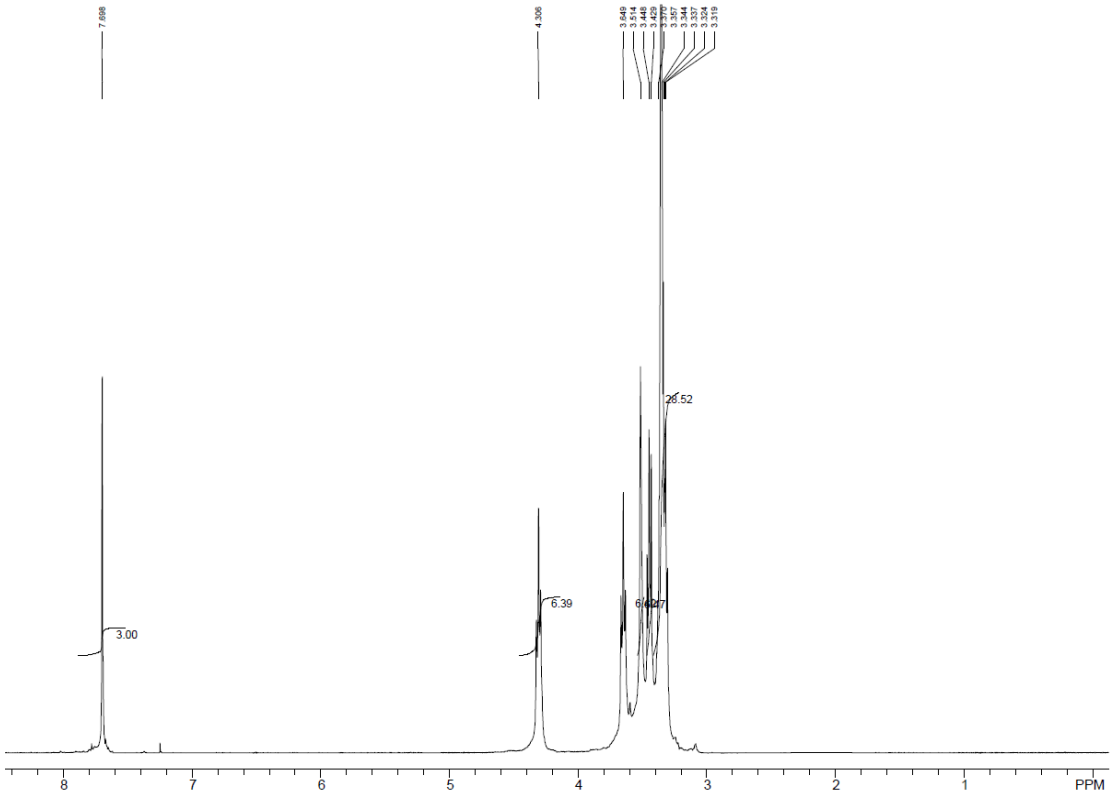
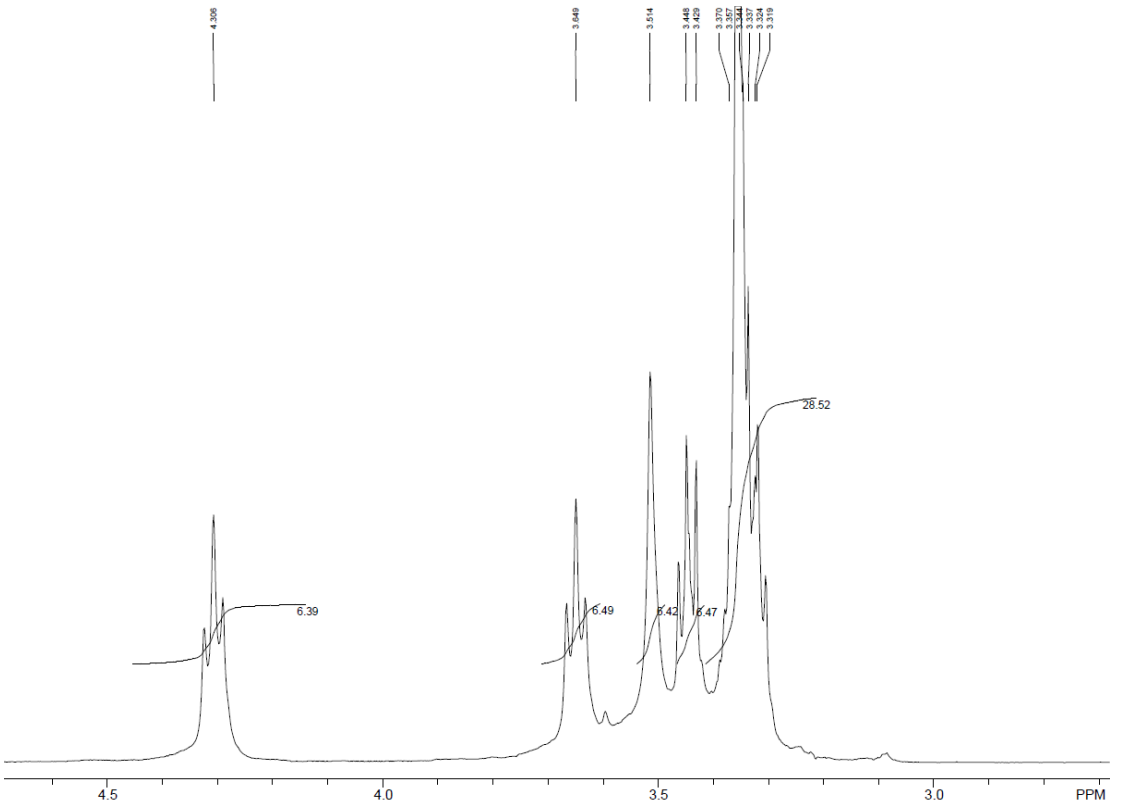


To a stirred solution of tripropargylamine (**7**, 250 mg, 1.91 mmol) in CH_3CN (2.50 mL) was treated sequentially with the azide **8** (1.50 g, 6.87 mmol), 2,6-lutidine (0.735 g, 6.87 mmol) and $Cu(MeCN)_4PF_6$ (27.7 mg, 0.070 mmol). After the mixture was stirred at room temperature for 24 h, the solvent was evaporated, and the residue was purified by flash chromatography (ethyl acetate/methanol 9:1) on silica gel to give **4** (930 mg, 1.18 mmol, 62%) as a light brown viscous liquid. ^{1}H NMR (300 MHz, $CDCl_3$): 7.69 (s, 3 H), 4.30 (t, 6 H, J = 5.0 Hz), 3.64 (t, 6 H, J = 5.0 Hz), 3.46 (s, 6 H), 3.44 (t, 6 H, J = 5.0 Hz), 3.37-3.30 (m, 30 H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 143.6, 124.4, 72.3, 70.2, 70.1, 70.0, 69.2, 61.1, 49.6, 47.2; MS (ESI) m/z calcd for $C_{33}H_{60}N_{10}O_{12}$: 788.4; found: 811.4 ($[M + Na]^+$).

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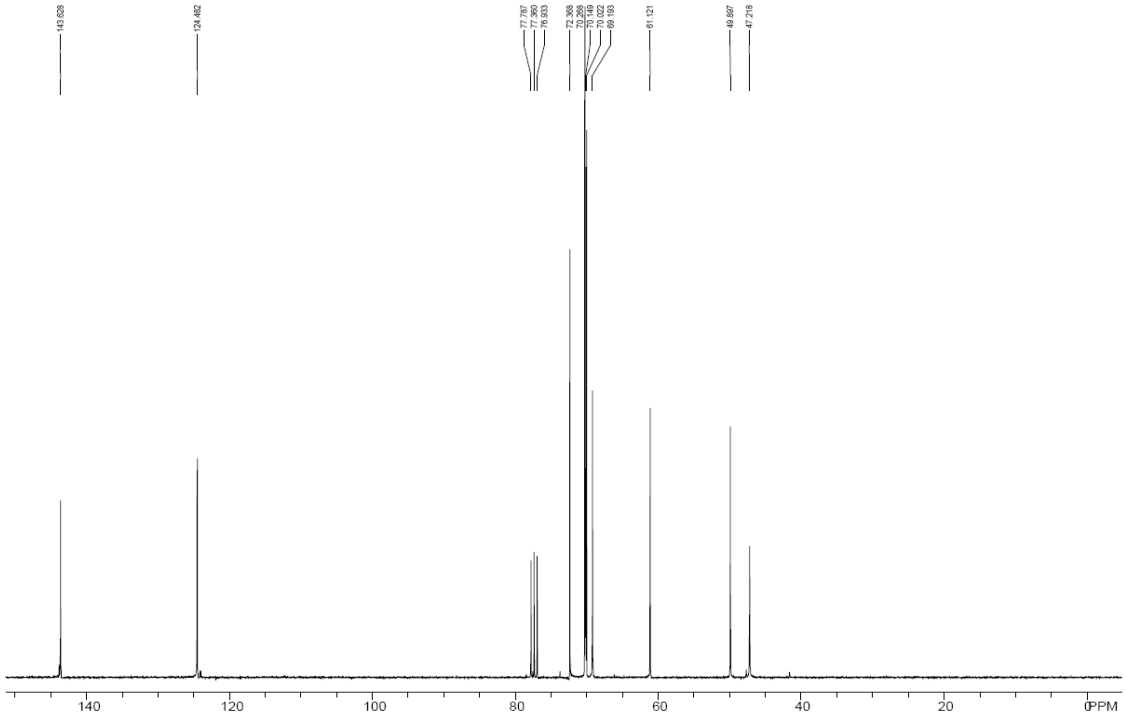
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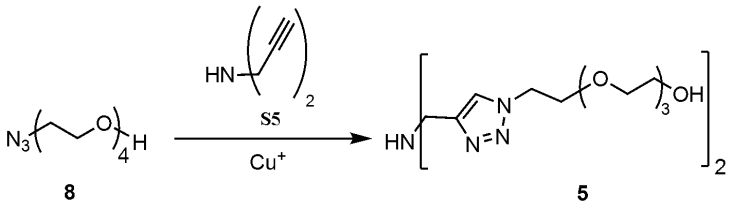
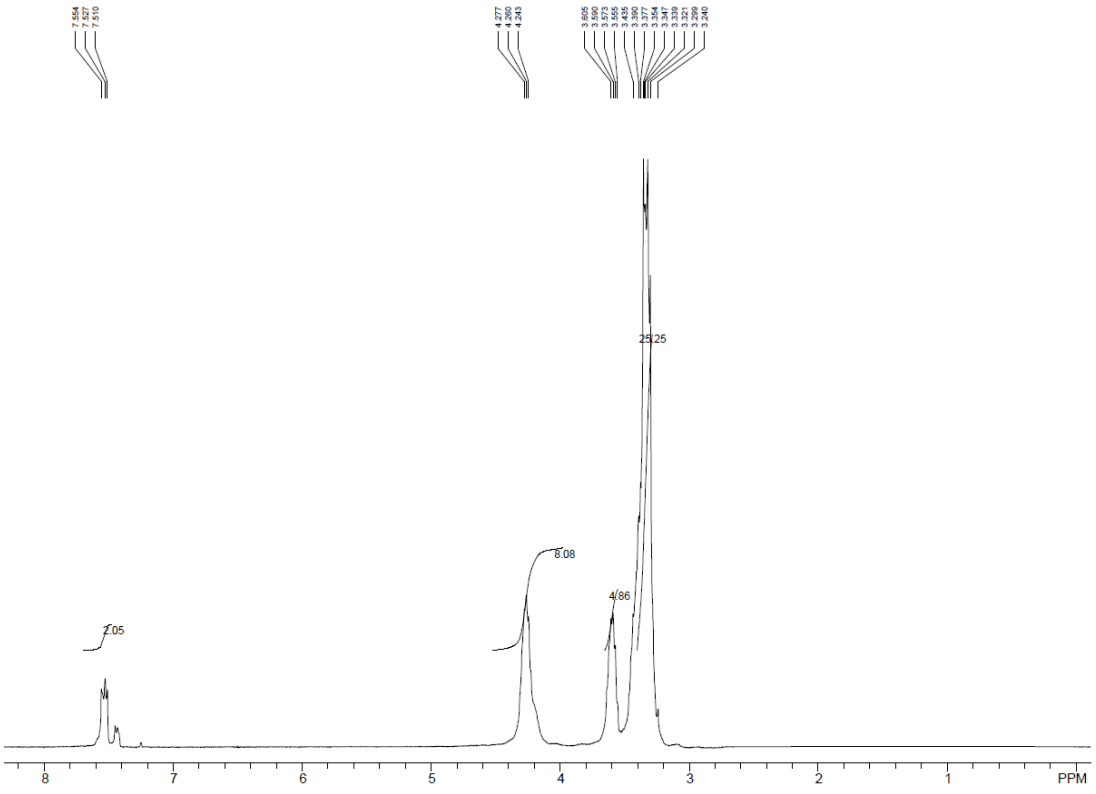
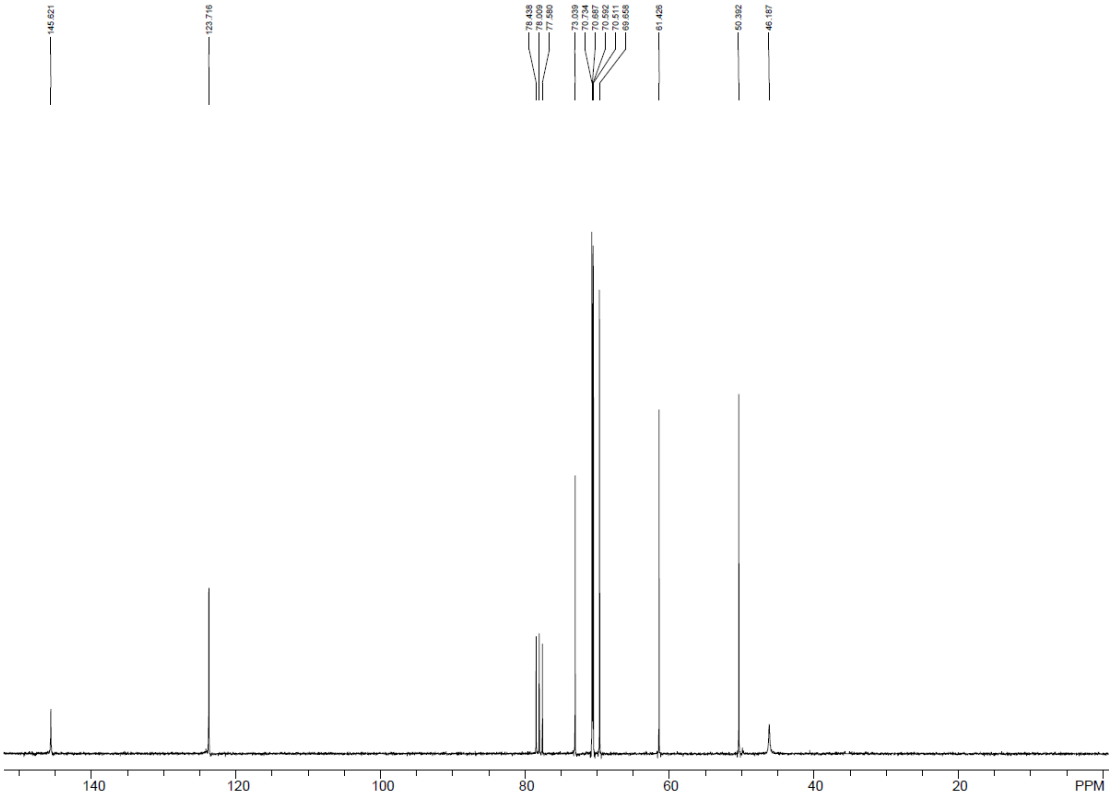
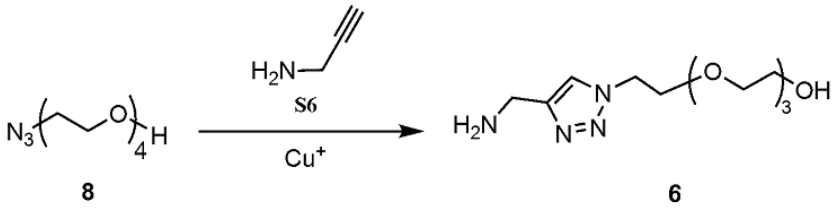


To a stirred solution of dipropargylamine (**S5**, 170 mg, 1.82 mmol) in CH_3CN (1.00 mL) was treated sequentially with azide **8** (954 mg, 4.36 mmol), 2,6-lutidine (466 mg, 4.36 mmol), and $Cu(MeCN)_4PF_6$ (17.6 mg, 0.040 mmol). The mixture was stirred at room temperature for 24 h, the solvent evaporated, and the residue purified by flash chromatography (ethyl acetate/methanol 9:1) on silica gel to give **5** (0.730 g, 1.34 mmol, 72%) as a light brown viscous liquid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.53 (s, 2 H), 4.26 (m, 8 H), 3.55 (m, 4 H), 3.65-3.24 (m, 24 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 145.6, 123.7, 73.0, 70.7, 70.6, 70.5, 69.7, 61.4, 50.4, 46.2; MS (ESI) *m*/*z* calcd for $C_{22}H_{41}N_7O_8$: 531.3; found: 555.3 ($[M + Na]^+$).

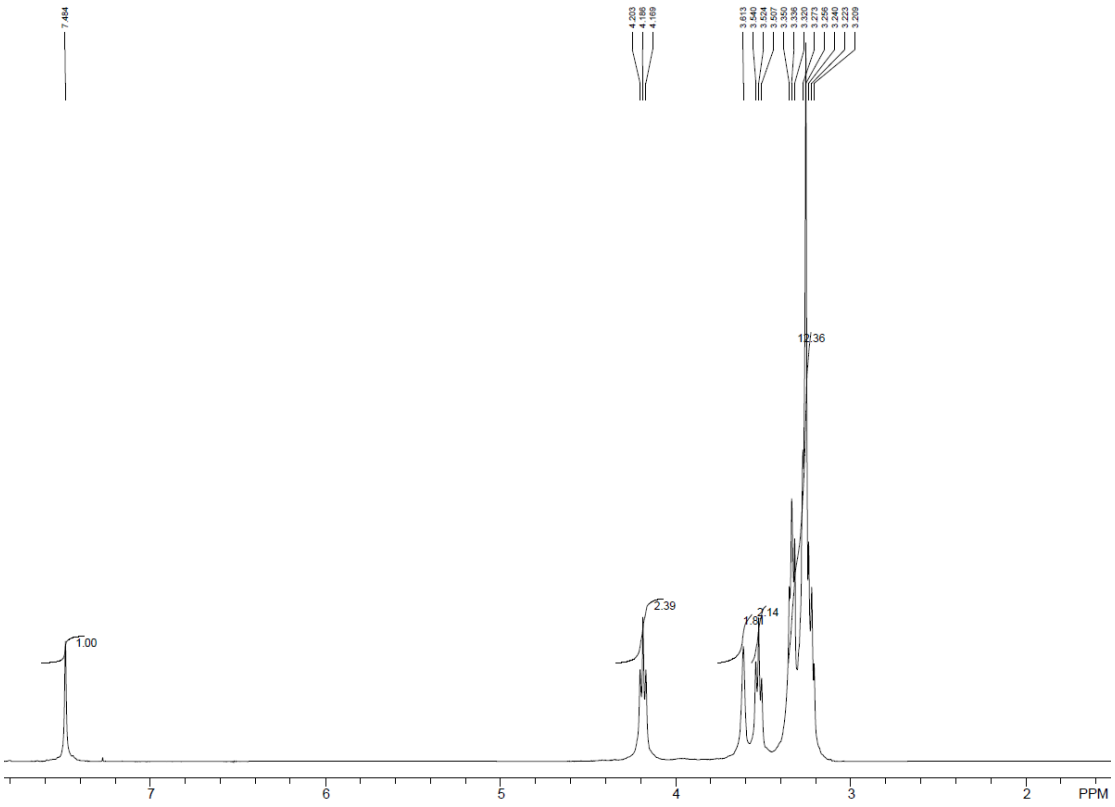






8 **6**

To a stirred solution of propargylamine (**S6**, 500 mg, 9.09 mmol) in CH_3CN (4.00 mL) was treated sequentially with azide **8** (2.38 g, 10.9 mmol), 2,6-lutidine (2.36 g, 10.9 mmol), and $Cu(MeCN)_4PF_6$ (40.9 mg, 0.110 mmol). The mixture was stirred at room temperature for 12 h, the solvent evaporated, and the residue purified by flash chromatography (ethyl acetate/methanol 9:1) on silica gel to give **6** (2.26 g, 8.27 mmol, 91%) as a light brown viscous liquid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.48 (s, 1 H), 4.18 (t, 2 H, J = 5.0 Hz), 3.61 (s, 2 H), 3.52 (t, 2 H, J = 5.0 Hz), 3.27-3.21 (m, 12 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 148.6, 122.7, 73.1, 70.7, 69.8, 61.3, 50.3, 37.4; MS (ESI) m/z calcd for $C_{11}H_{22}N_4O_4$: 274.3; found: 297.3 ($[M + Na]^+$).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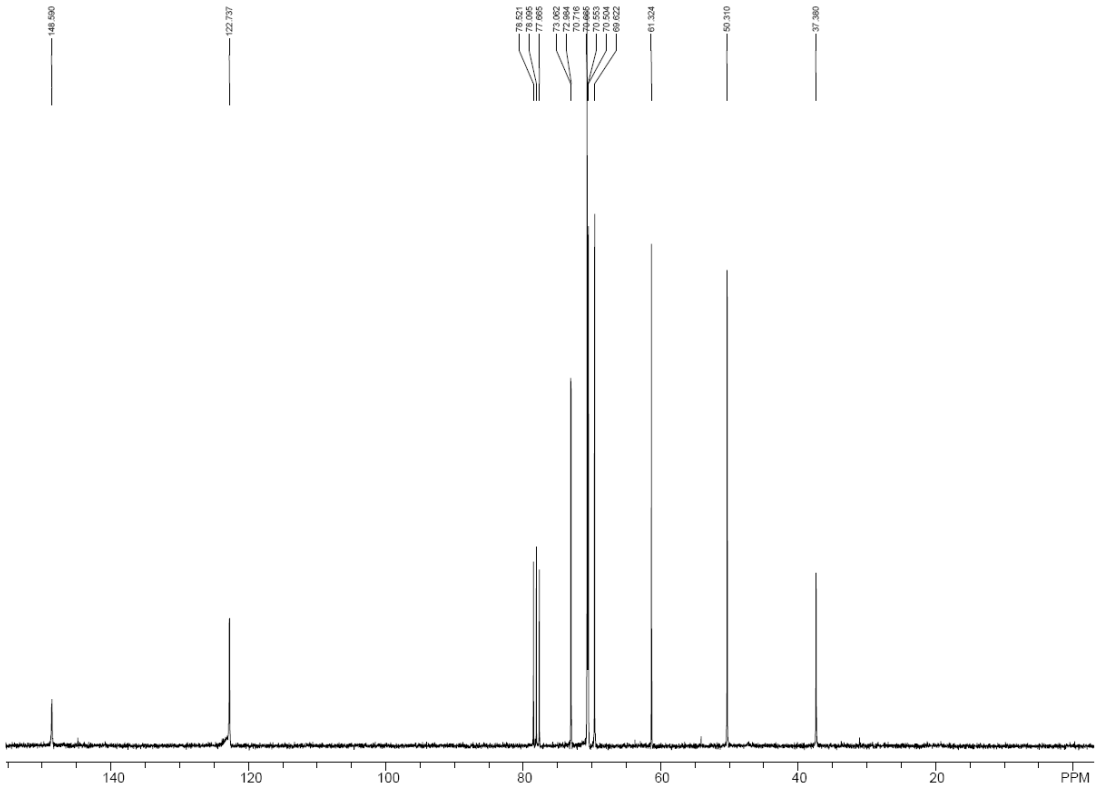
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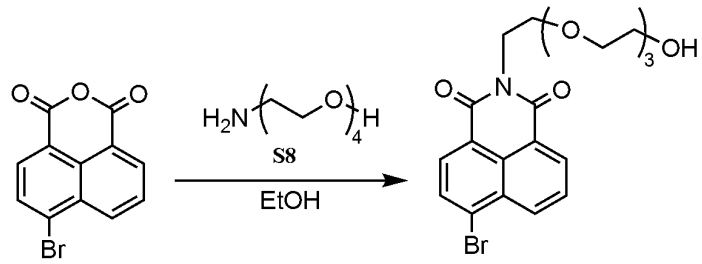
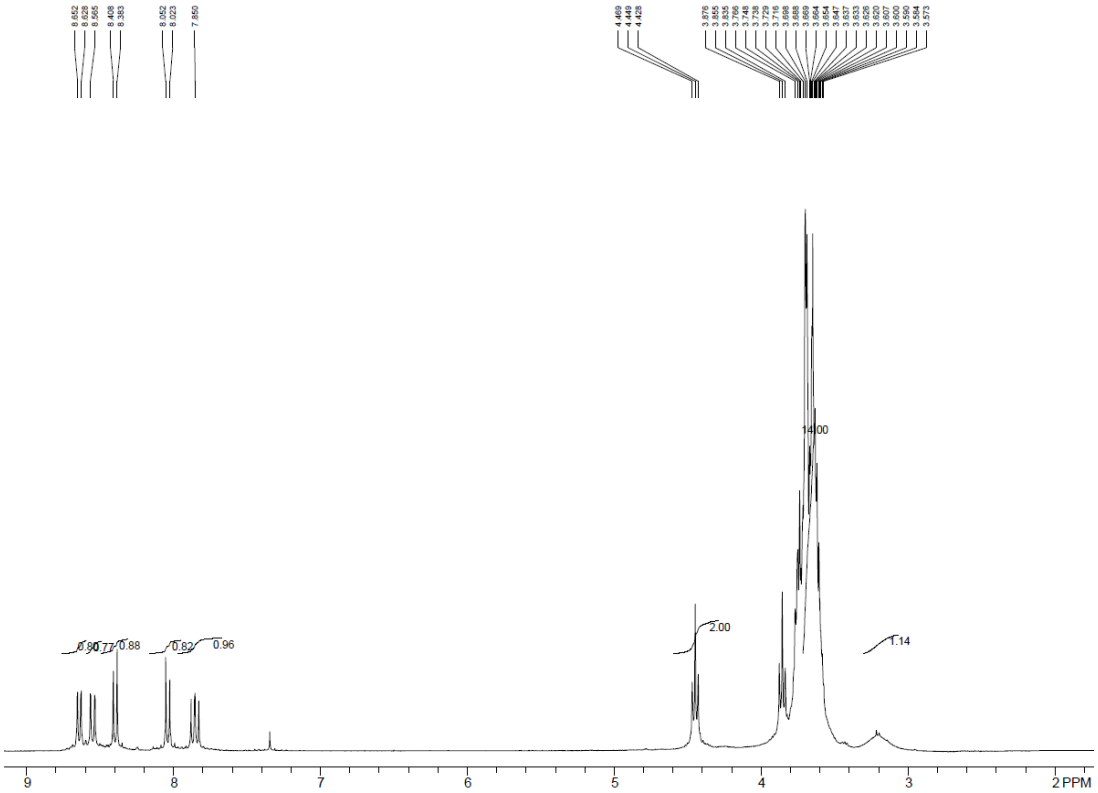
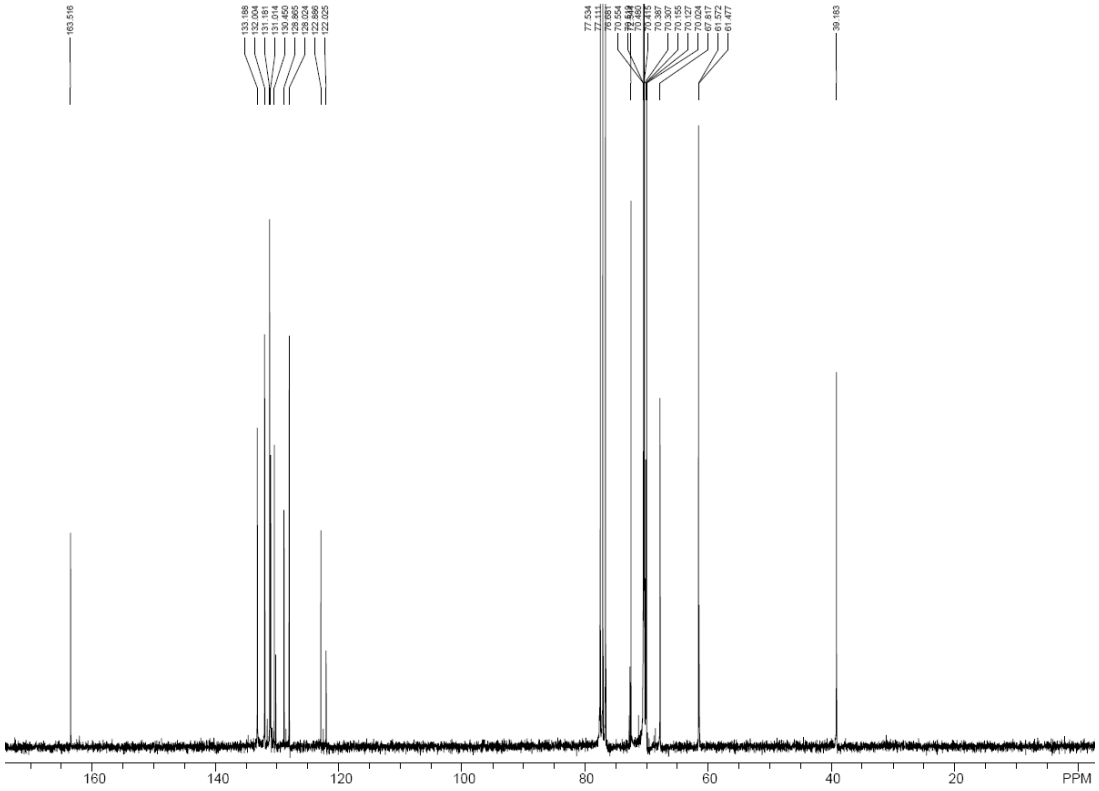
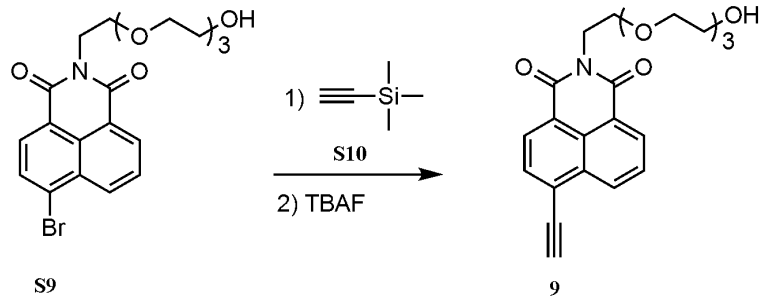


S7 **S9**

To a solution of anhydride **S7** (200 mg, 0.720 mmol) in ethanol (1.00 mL) was added amine **S8** (153 mg, 0.790 mmol) and the solution was refluxed for 12 h. Thereafter, the solvent was evaporated, and the residue purified by flash chromatography (ethyl acetate) on silica gel to give **S9** (0.220 g, 0.470 mmol, 67%) as a light brown semi-solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.65 (d, 1 H, J = 7.3 Hz), 8.56 (d, 1 H, J = 7.8 Hz), 8.40 (d, 1 H, J = 8.3 Hz), 8.05 (d, 1 H, J = 8.0 Hz), 7.85 (m, 1 H), 4.44 (t, 2 H, J = 6.2 Hz), 3.87-3.57 (m, 14 H), 3.20 (bs, 1 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 163.5, 133.2, 132.0, 131.2, 131.0, 130.5, 128.9, 128.0, 122.9, 122.0, 72.5, 70.5, 70.4, 70.3, 70.2, 70.1, 70.0, 67.8, 61.6, 39.2; MS (ESI) m/z calcd $C_{20}H_{22}BrNO_6$: 452.3; found: 475.3 ($[M + Na]^+$).



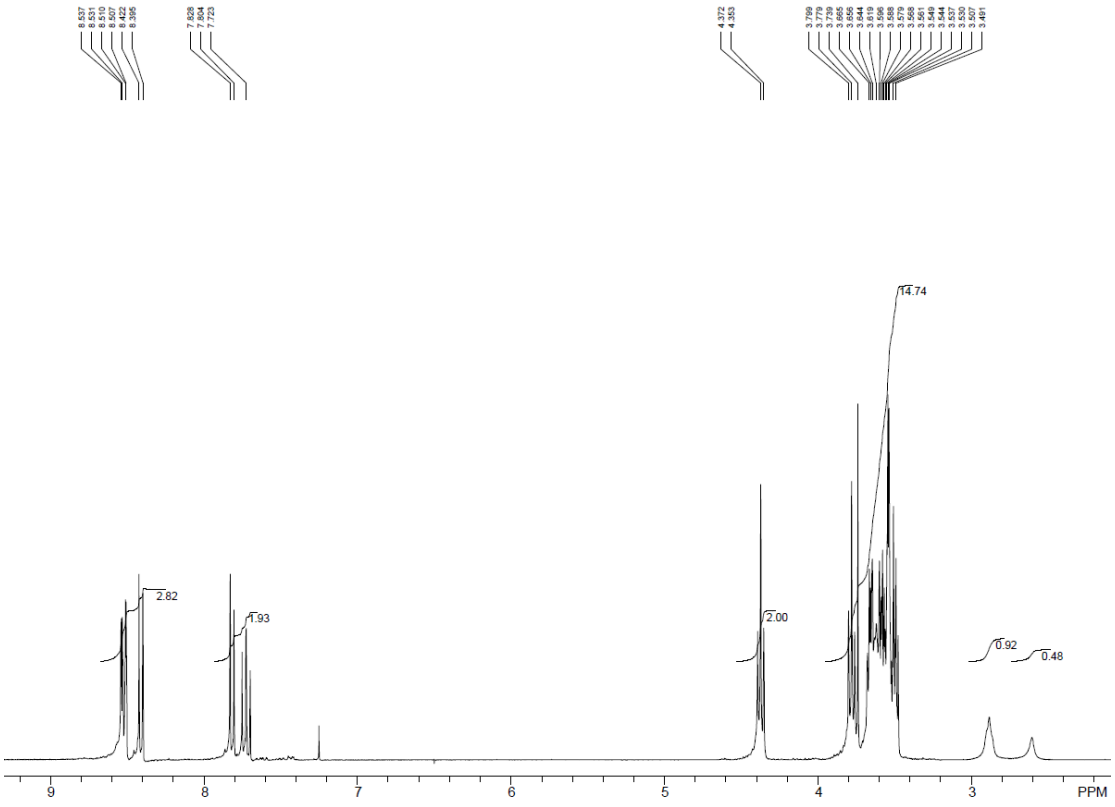


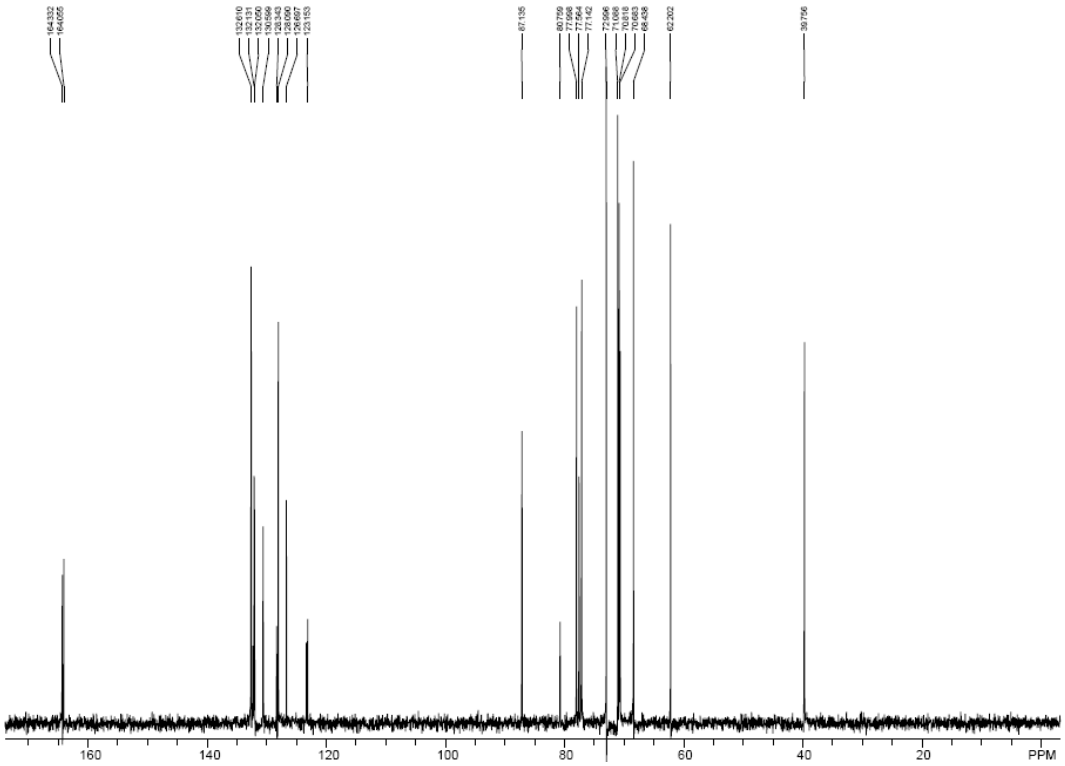


S9 **9**

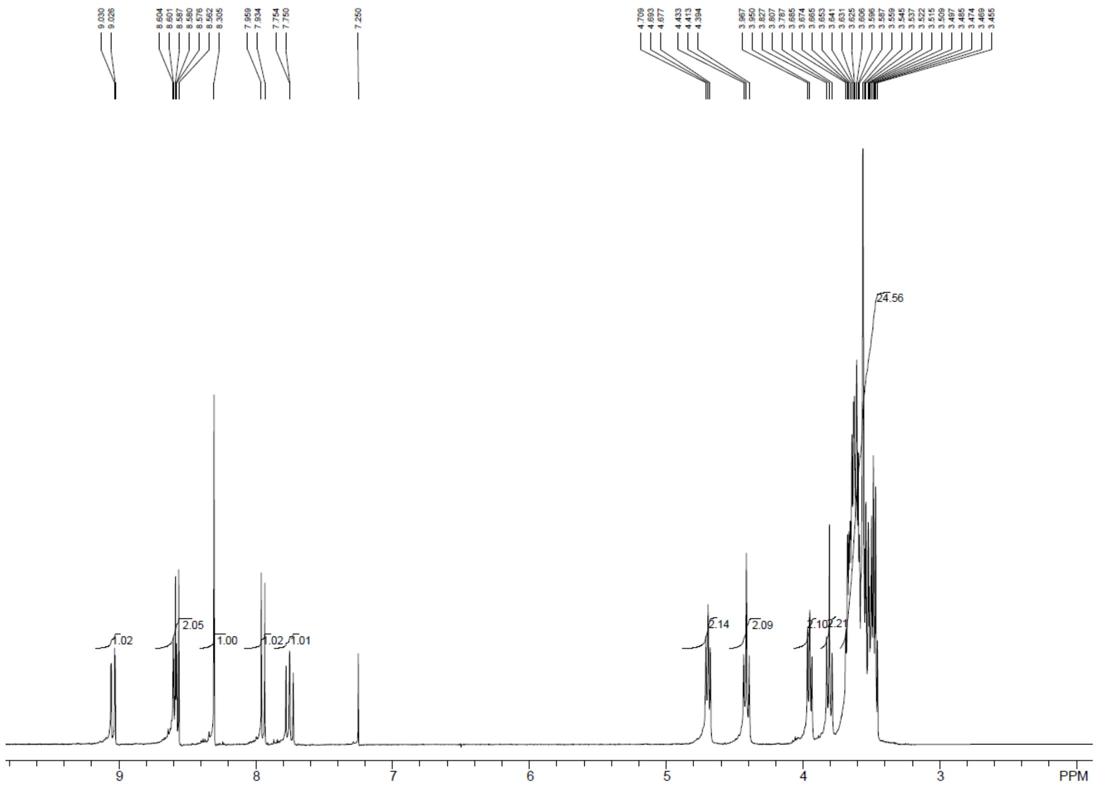
To a solution of bromonaphthalimide derivative, **S9**, (561 mg, 1.54 mmol) in THF (10.0 mL) was added tetrakis(triphenylphosphine)palladium (180 mg, 0.156 mmol), CuI (60.0 mg, 0.320 mmol), trimethylsilylacetylene (**S10**) (1.08 mL, 7.64 mmol), and *N*,*N*-diisopropylethylamine (1.00 mL, 5.64 mmol) under nitrogen atmosphere. The mixture was stirred at room temperature overnight. The reaction mixture was diluted with ethyl acetate (100 mL), washed with saturated NH_4Cl solution, dried over Na_2SO_4, and evaporated. The residue was purified by column chromatography (ethyl acetate) on silica gel to give the corresponding trimethylsilyl compound (180 mg). To the solution of this compound (180 mg) dissolved in MeOH (25.0 mL) was added 1.00 M tetrabutylammonium fluoride (TBAF) in THF (2.00 mL), and the mixture stirred at 60 °C for 15 min. The reaction mixture was diluted with water (50.0 mL), and the precipitate collected by filtration. The resulting solid was purified by flash column chromatography (ethyl acetate) using silica to afford **9** as a yellow solid (143 mg, 0.460 mmol, 30%) ^{1}H NMR (300

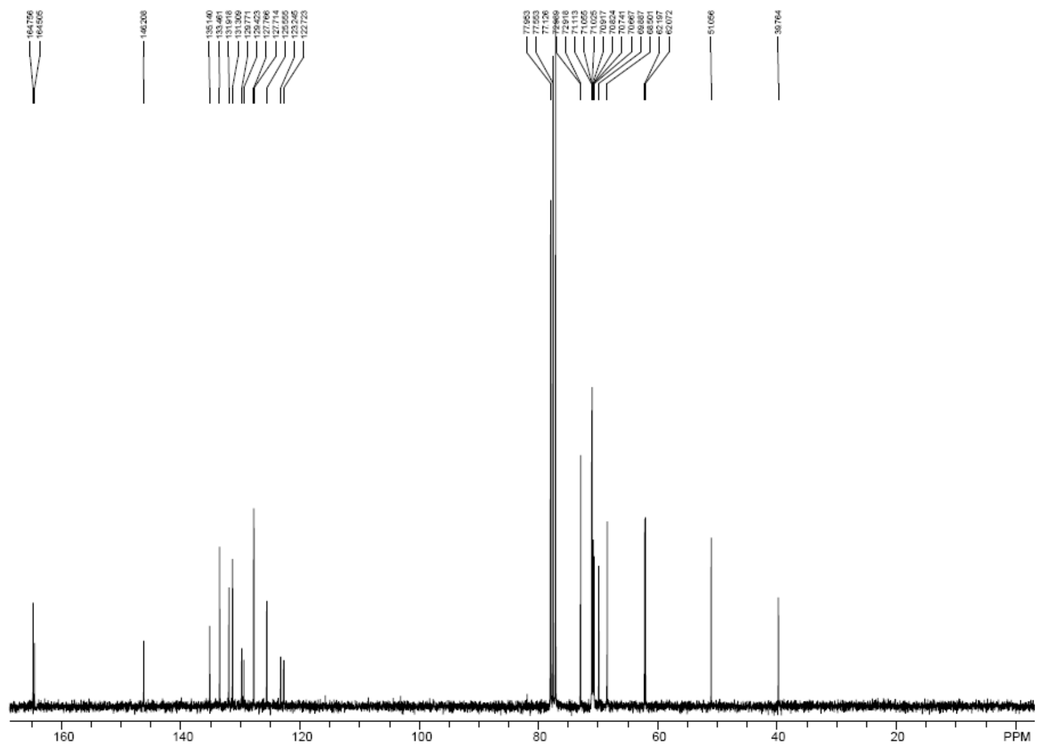
MHz, $CDCl_3$) δ 8.53 (d, 1 H, J = 7.1 Hz), 8.51 (d, 1 H, J = 8.2 Hz), 8.42 (d, 1 H, J = 8.2 Hz), 7.82 (d, 1 H, J = 8.2 Hz), 7.72 (m, 1 H), 4.37 (t, 2 H, J = 6.2 Hz), 3.79-3.49 (m, 14 H), 2.89 (bs, 1 H), 2.61 (bs, 1 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 164.3, 164.0, 132.6, 132.1, 130.6, 128.3, 128.0, 126.7, 123.2, 87.1, 80.8, 72.9, 71.0, 70.8, 70.7, 68.4, 62.2, 39.8; MS (ESI) m/z calcd $C_{22}H_{23}NO_6$: 397.1; found: 420.2 ($[M + Na]^+$).





To a stirred solution of alkyne **9** (100 mg; 0.430 mmol) in CH_3CN (1.00 mL) was treated sequentially with azide **8**[23] (178 mg, 0.430 mmol), ligand **4** (0.009 g, 11.1 μmol), and $Cu(MeCN)_4PF_6$ (2.10 mg, 5.59 μmol). After the mixture was stirred at room temperature for 12 h, the solvent was evaporated, and the residue purified by flash chromatography (ethyl acetate/methanol 9:1) on silica gel to give **10** (306 mg, 0.320 mmol, 97%) as a light brown viscous liquid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.03 (d, 1 H, J = 8.2 Hz), 8.60-8.56 (m, 2 H), 8.31 (s, 1 H), 7.96 (d, 1 H, J = 7.8 Hz), 7.75 (m, 1 H), 4.71 (t, 2 H, J = 4.5 Hz), 4.41 (t, 2 H, J = 5.9 Hz), 3.95 (t, 2 H, J = 4.5 Hz), 3.81 (t, 2 H, J = 5.9 Hz), 3.69-3.46 (m, 24 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 164.7, 164.5, 146.2, 135.1, 133.5, 131.9, 131.3, 129.8, 129.4, 127.8, 125.6, 123.2, 122.7, 72.9, 71.1, 71.0, 70.9, 70.8, 70.7, 70.6, 69.9, 69.5, 62.2, 62.1, 51.1, 39.8; MS (ESI) m/z calcd $C_{35}H_{50}N_4O_{13}$: 734.3; found: 757.3 ($[M + Na]^+$).





B: Evaluation of the Co-catalysts under Aerobic Conditions

Reactants were freshly prepared in 50.0 mM (pH 7.2) HEPES buffer (Invitrogen). They were added to and mixed with a pipette in a 96-well plate at a fixed concentration of the alkyne **9** (0.500 mM) and azide **8** (1.00 mM), whereas the concentrations of Cu(I), ligand, and ascorbic acid were varied. Each ligand was tested in a similar way. The ratio of ligand/Cu(I) was always fixed at 2:1. While in the 96 well plate, each row was loaded with different Cu(I) concentration, every column was loaded with different ascorbic acid concentration. The overall volume in each well of the plate was 250 μL containing a solution of the azide **8** (1.00 mM), alkyne (0.500 mM) $CuSO_4$ (1.00-0.125 mM), ligand (**1c, 4, 5**) (2.00-0.250 mM), and sodium ascorbate (5.00-1.00 mM). The reactions were allowed to stand at room temperature and 6.00 μL was taken out at regular time interval, diluted 60 times with the same buffer and then transferred to another 96-well microtiter plate for measurement of the fluorescence intensity at λ_{ex} = 365 nm and λ_{em} = 420 nm using a Fusion plate reader (Perkin-Elmer/Packard, Wellesley, MA). The concentration of the product **10** with a maximum λ_{ex} = 358 nm and λ_{em} = 422 nm was then obtained after correlating the measured fluorescence intensity with a calibration curve (Figure S1) and multiplying the value by 60 (since the reaction mixture was diluted 60 times before the measurement). The calibration curve was obtained from solutions of **10** 50.0 mM (pH 7.2) HEPES buffer.

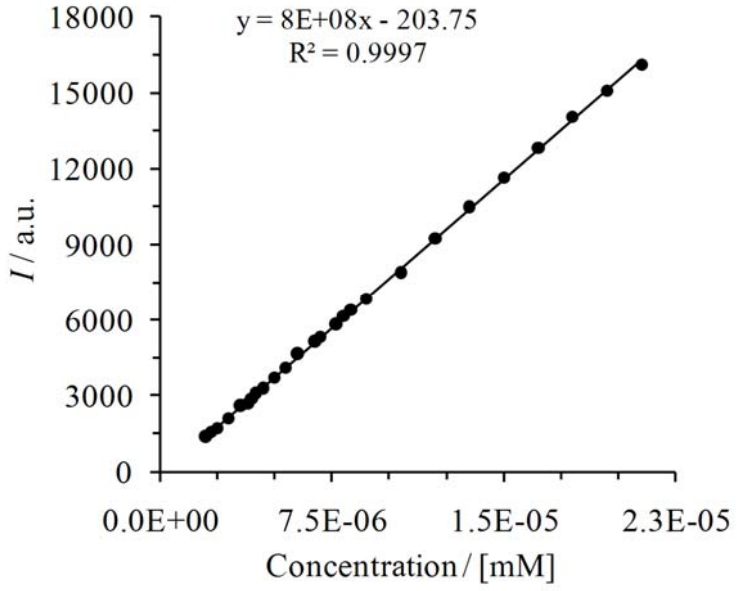


Figure S1 Concentration-dependent fluorescence (λ_{ex} = 360 nm, λ_{em} = 420 nm) intensities (*I*) of **10** in 50.0 mM (pH 7.2) HEPES buffer.

C: Evaluation of the Co-catalysts under Anaerobic conditions

Stock solutions each of the following components, $CuSO_4$ (0.625 mM), ligand (**3**, **4**, **5**, **6** and **1c**, 1.25 mM), the 1,8-naphthalimide alkyne derivative **9** (2.50 mM), azide **8** (5.00 mM) and sodium ascorbate (6.25 mM) in 50.0 mM HEPES buffer were prepared, and then the sequence of freeze-vacuum-filling N_2-thawed was repeated 15 times in a Schlenk tube. In the above order, an aliquot of 0.200 mL of each solution was taken with a micro syringe under a positive nitrogen pressure to prevent any air seepage and added into a schlenk tube under nitrogen. The time was recorded after the addition of ascorbic acid. A 10.0 μL of the reaction mixture was withdrawn at specified time duration from the Schlenk tube and injected into a silica gel HPLC column (Monolithic Onyx, Phenomenex), and eluted with a gradient (EtOAc to 10% MeOH in EtOAc in 30 min) using a Prominence HPLC (Shimadzu). The peaks were recorded at λ_{ex} = 360 nm *via* SPD-M20A Shimadzu Prominence diode array detector. The yields were obtained using the following calibration curve (Figure S2) correlating the peak area with the concentrations of **10**.

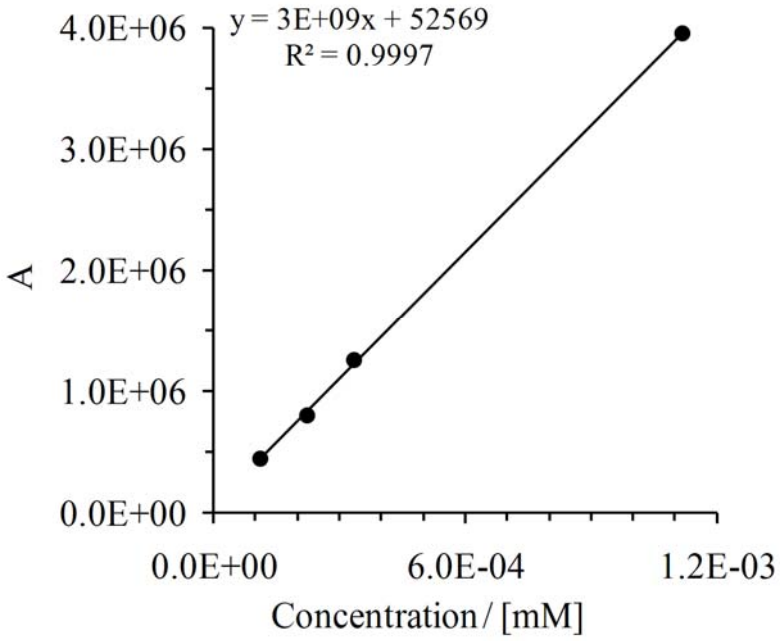


Figure S2 HPLC peak area (A) (λ_{ex} = 360 nm) of **10** *vs* concentration.

D: Determination of the Amount of Active Carbonyl on BSA

For measuring the amount of carbonyl groups formed on BSA after subjecting it to CuAAC reaction conditions, the following stock solutions were prepared. For condition (a, see main text and Figure 2), the following solutions were made from 0.1 M PBS buffer (pH 7.0): (1) 0.6 mM $CuSO_4$, (2) 15 mM sodium ascorbate, and (3) 3 mM of the ligand **4**.. For condition (b), the following solutions were made from 0.1 M PBS buffer (pH 7.0) containing 5% DMSO: (1) 0.8

mM $CuSO_4$, (2) 20 mM sodium ascorbate, (3) 4 mM of the ligand **4**, and (4) 40 mM amino guanidine. The stock solutions prepared for conditions (a) and (b) were mixed (250 μL each) in an Eppendorf tube. A 2.5 μL of the mixed solution was then added to a 96-well plate, each well already contained 2.5 μL of the fully reduced BSA standard (Cell Biolabs Inc., 1 mg/mL). The mixtures were left at ambient conditions for a duration specified in Fig 2. A volume of 4 μL was removed from the 96-well plate and diluted with 126 μL of 0.1 M PBS buffer (pH 7.0) and then purified with size exclusion chromatography using PD SpinTrap™ G-25 (GE Healthcare) columns. The resulting solution was diluted to 10 μg/mL by adding 70 μL of 0.1 M PBS buffer (pH 7.0). For measuring the amount of carbonyl generated by conditions (c) and (d) in an anaerobic chamber (Rigid Anaerobic Chamber running on a mixture of N_2 with 5 % H_2, equipped with an Oxygen and Hydrogen Analyzer, Coy Laboratory Inc.), the following stock solutions were prepared in 50.0 mM HEPES buffer (pH 7.2, Invitrogen): $CuSO_4$ (0.75 mM), sodium ascorbate (7.5 mM), and ligand **5** (1.5 mM) by weighing the requisite amount of solid and diluting it inside the chamber with 50.0 mM (pH 7.2) HEPES buffer (invitrogen) (which was previously exposed to 5 freeze-thaw pump cycles and stored inside the anaerobic chamber) having the requisite amount of oxygen (see Fig. 2). A 250 μL of the above three reactants were mixed in an Eppendorf tube. A 2.5 μL of the prepared solution was then added to a 96 well-plate which already contained 2.5 μL of fully reduced BSA (Container holding1 mg/mL of BSA was pre flushed with nitrogen and was stored in anaerobic chamber for use at least 12 h before). The reaction was left at ambient conditions for specified time (Fig 2.) before it was purified by taking a volume of 4 μL from each well, and diluted with 126 μL of 50 mM HEPES buffer (pH 7.2) and immediately purified with size exclusion chromatography using PD SpinTrap™ G-25 (GE Healthcare) columns. The resulting solution was diluted to 10 μg/mL by adding 70 μL of 50 mM HEPES buffer (pH 7.2). All the processes up until this step were carried out inside the anaerobic chamber. The samples were immediately proceeded to the determination of the amount of carbonyl on the BSA with an OxiSelect™ protein carbonyl ELISA kit (Cell Biolabs Inc.) using the following protocol provided by the vendor.

1. BSA samples were diluted to 10 μg/mL in 1X PBS.

2. An aliquote of 100 μL solution of each BSA sample (10 μg/mL), including the oxidized BSA standards, was added to the 96-well protein binding plate and incubated at 37 ºC for 2 h.

3. Each well was washed 3 times with 250 μL 1X PBS. After the last wash, wells were emptied and tapped on an absorbent pad or paper towel to remove excess the wash solution.

4. A 100 μL DNPH working solution was added to each well and incubated for 45 minutes at room temperature in the dark. The DNPH working solution was made by weighing out 5.0 mg of DNPH, and dissolving it in DNPH diluent to 1 mg/mL. This DNPH stock solution was stable for one week and was stored in the dark at 4 ºC. Based on the number of tests, appropriate amount of working solution of DNPH was freshly prepared by diluting the 1 mg/mL stock DNPH solution

to 0.04 mg/mL in DNPH diluent. Care was taken to use the sample immediately as the diluted sample was unstable.

5. Wells were washed with 250 µL of 1X PBS/Ethanol (1:1, v/v) with incubation on an orbital shaker for 5 min. The process was repeated 5 times, with aspiration between each step. After the last wash, wells were emptied and tapped on absorbent pad or paper towel to remove the excess wash solution. This was followed by washing twice with 250 µL of 1X PBS.

6. A 200 µL blocking solution was added per well and incubated for 2 h at room temperature on an orbital shaker.

7. The wells were washed 3 times with 250 µL of 1X wash buffer and thorough aspiration was carried out between each wash. After the last wash, the wells were emptied and tapped on absorbent pad or paper towel to remove the excess of 1X wash buffer.

8. A 100 µL solution of the diluted anti-DNP antibody was added to each well and incubate for 1 hour at room temperature on an orbital shaker. Each well was washed 3 times according to step 7 (Anti-DNP antibody was prepared immediately before use by diluting the anti-DNPH antibody to a ratio of 1:1000 with 1X blocking solution.)

9. A 100 µL solution of diluted HRP conjugated secondary antibody was added to each well and incubated for 1 hour at room temperature on an orbital shaker. Each well was washed 5 times according to step 7 above. (HRP conjugated secondary antibody was prepared immediately before use by diluting the HRP conjugated secondary antibody to a ratio of 1:1000 with 1X blocking solution.)

10. The substrate solution was warmed to room temperature. 100 µL of substrate solution was added to each well, including the blank well and incubated at room temperature on an orbital shaker for 2 min.

11. The enzymatic reaction was stopped by adding 100 µL of stop solution to each well. Results were read immediately using a plate reader (FLUOstar OPTIMA, BMG LABTECH**)** at 450 nm as the primary wavelength.

E: References

1. P. S. Shirude, V. A. Kumar and K. N. Ganesh, *Eur. J. Org. Chem.*, 2005, 5207-5215.
2. P. Besenius, P. A. G. Cormack, R. F. Ludlow, S. Otto and D. C. Sherrington, *Chem. Commun.*, 2008, 2809-2811.