Electronic Supplementary Material (ESI) for Physical Chemistry Chemical Physics.

Electronic Supplementary Information

Electron Transfer Rate Analysis of a Site-Specifically Wired Copper Oxidase

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Supporting figures

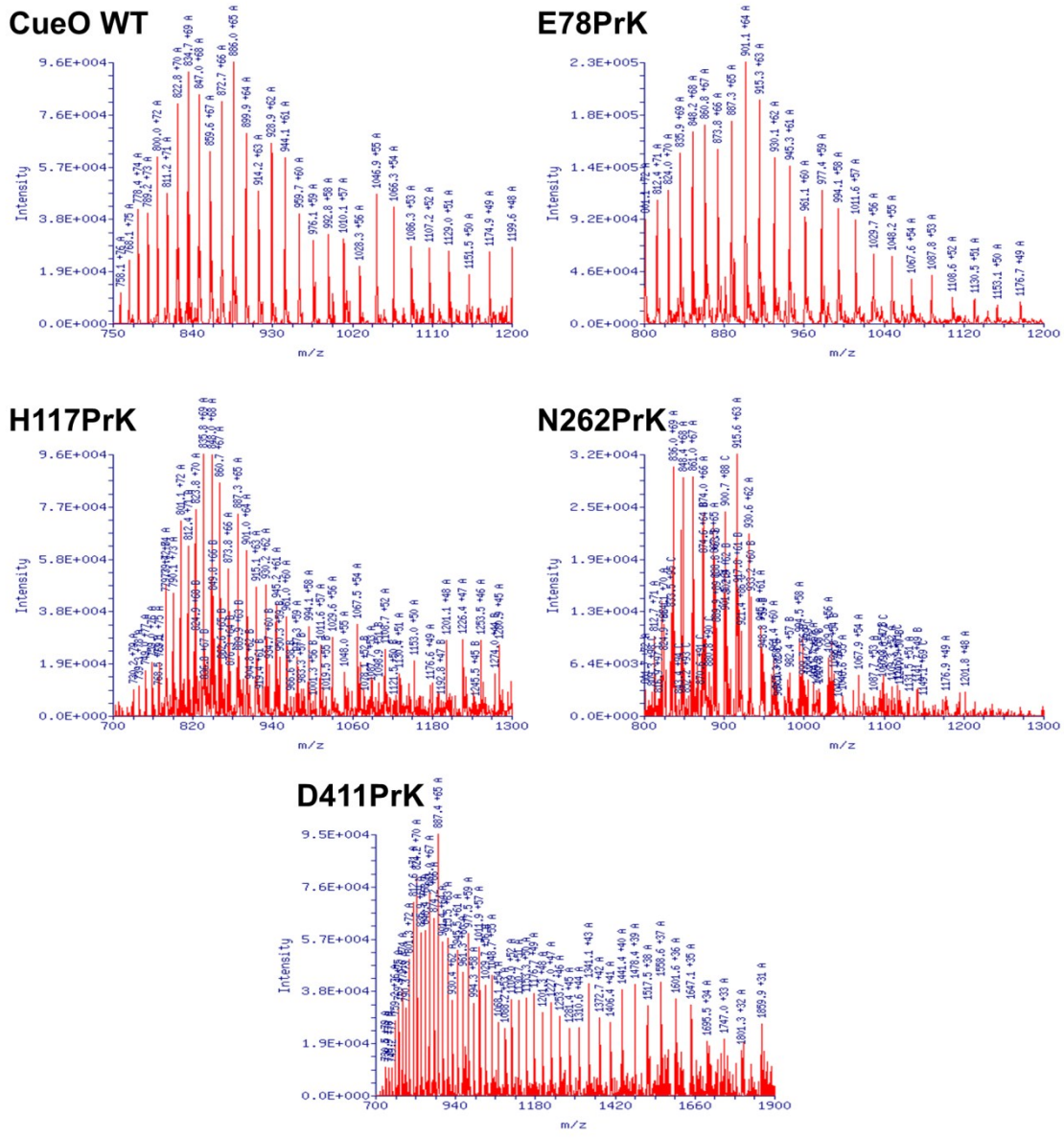


Figure S1: Mass spectra of CueO mutants. Raw mass spectra of each CueO mutant.

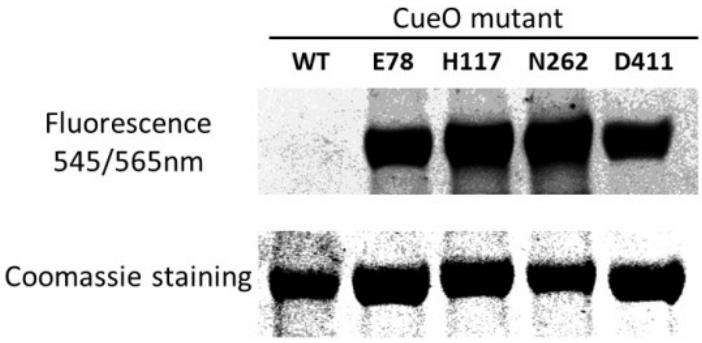


Figure S2: fluorescent SDS-PAGE of Azide-fluor 545 labeled purified CueO mutants. Only CueO mutants with incorporated PrK react with the azide modified fluorescent label, generating a fluorescent band on the gel. Coomassie staining confirms the presence of labeled/unlabeled CueO in all samples.

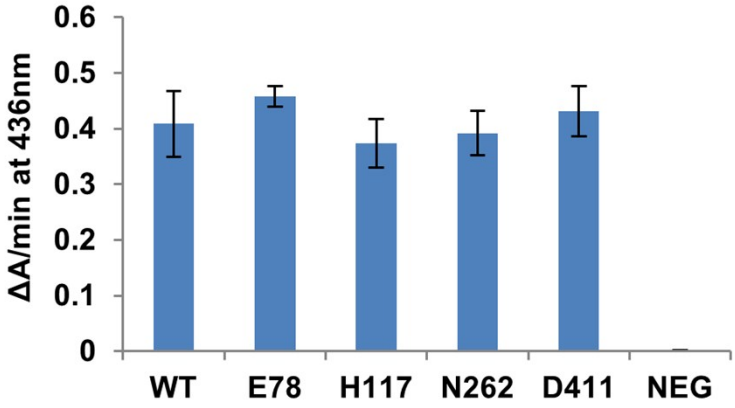


Figure S3: o-phenylenediamine (OPD) biochemical activity assay of CueO mutants.
Biochemical activity assay using OPD as a CueO substrate. Oxidation of OPD by CueO was monitored by the change in absorbance at 436 nm. Negative control was done by addition of buffer instead of enzyme solution. Assay was performed in 50 mM acetate buffer pH=4 at 37°C.

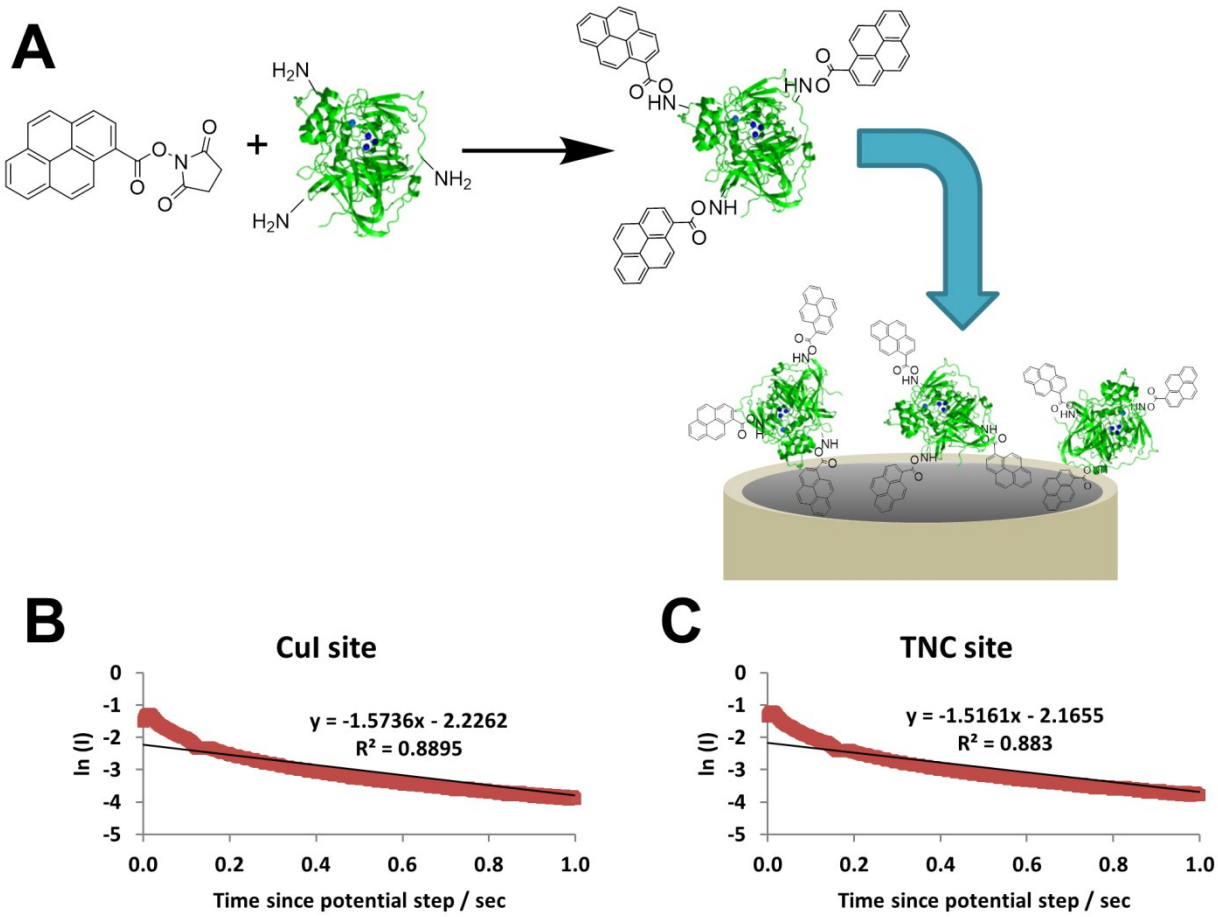


Figure S4: Performance of non-specifically immobilized CueO modified electrode.
(A) Non-specific immobilization through native amine residues on the enzyme surface produced various orientations of the enzyme towards the electrode support. Panels (B) and (C) show a representative linearized signal from non-specifically modified electrode using PSE. The linearized decay patern is far from being linear, suggesting that more than one specie contributes to the generated current. The average ET coefficients for the CuI and TNC sites are 1.574 s^{-1} and 1.516 s^{-1}, respectively. Measurements were performed in 0.1 M acetate buffer pH=4.0.

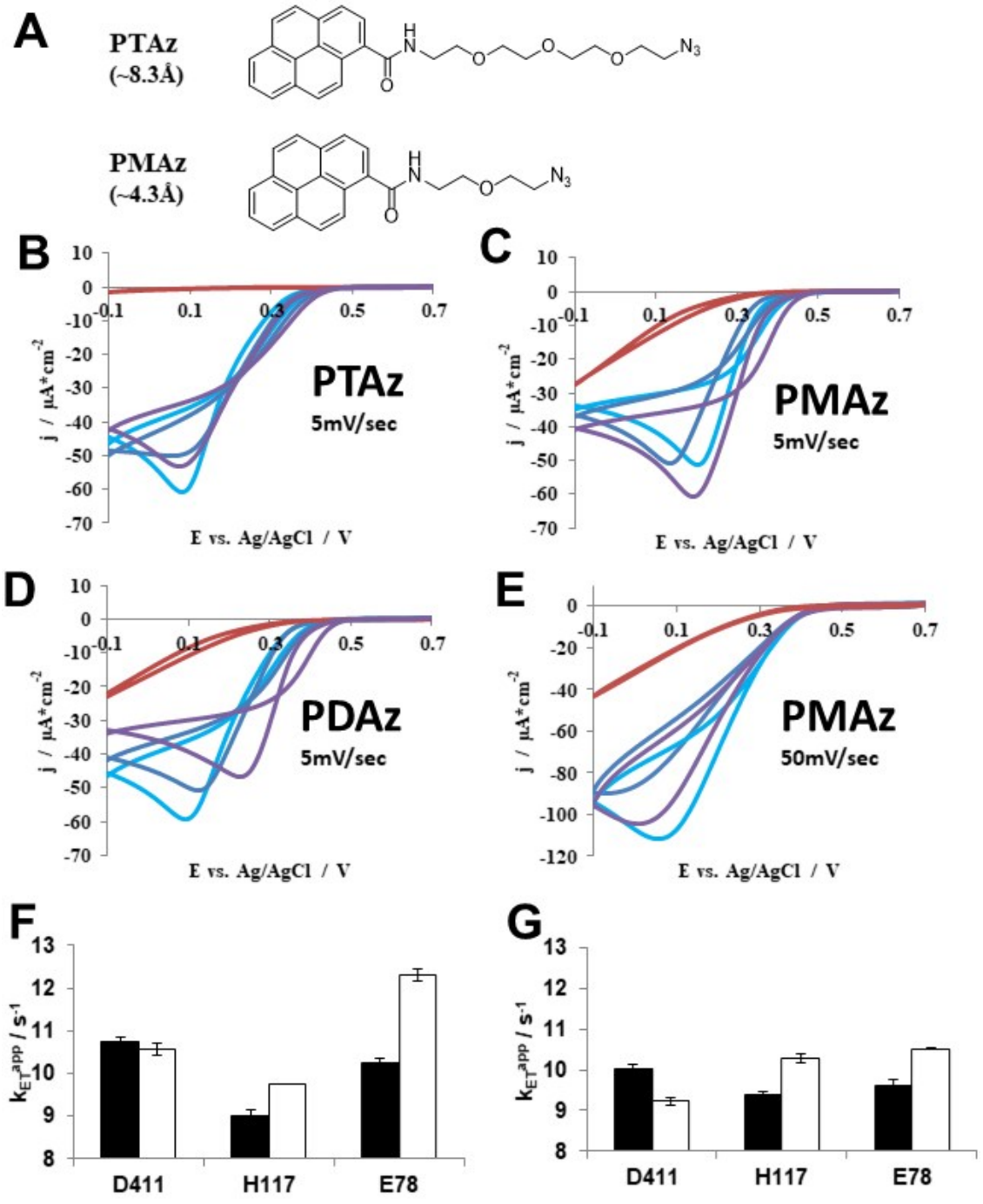


Figure S5: The effect of linker length on ET kinetics.

Panel (A) shows the long and short linkers, PTAz and PMAz, respectiviely, with their approximated fully stretched length. Cyclic voltammograms of CueO mutants in the presence of oxygen, showing the cathodic catalytic current generated using PTAz (B) PMAz (C) and PDAz (D). CVs of wired E78PrK (cyan), H117PrK (blue), D411PrK (purple) and the distant mutant N262PrK (red). The crossing of the voltammograms in the slow scan rate is eliminated when scan rate increases to 50 mV/sec (E). Panels (F) and (G) show the apparent ET rate constants measured for mutants immobilized using PTAz and PMAz, respectively. ET rates were measured for the CuI (black bars) and TNC (white bars) redox centers. Cyclic voltammograms were generated at a scan rate of 5 mV/sec. All measurements were performed in 0.1 M acetate buffer pH=4.0.

Supporting tables:

Table S1:

Mutant	Calculated mass	Deconvoluted mass
WT CueO	57,522Da	57,526.5 ± 4.9Da
E78PrK	57,603Da	57,603.4 ± 4.2Da
H117PrK	57,595Da	57,600.0 ± 7.6Da
N262PrK	57,618Da	57,621.8 ± 10.3Da
D411PrK	57,617Da	57,617.1 ± 7.8Da

Table S1: Mass spectra of CueO mutants. Calculated and deconvoluted experimental mass of each CueO mutant.

Supplementary methods

Mass Spectrometry of purified proteins

The purified proteins and ncAA incorporation were verified by mass spectrometry. The purified protein was analyzed by LC-MS (Finnigan Surveyor Autosampler Plus/LCQ Fleet (Thermo Scientific, Waltham, MA), using Chromolith® FastGradient RP-18 endcapped 50-2 HPLC column (Merck Millipore, Billerica, MA, USA) for sample preparation. The results were analyzed using Xcallibur (Thermo, Waltham, MA, USA) and Promass (Novatia LLC, Newtown, PA, USA) software.

Fluorescent gels

PrK mutants of CueO were labeled with an azide functionalized fluorescent label (Azide-fluor 545, Sigma-Aldrich, Rehovot, Israel) using click chemistry. The reaction mixture (50 µL) contained 20 µL of the crude lysate, 0.1 M sodium phosphate buffer pH=7.0 and 100 µM of the fluorescent label. $CuCl_2$ and Tris(3-hydroxypropyltriazolylmethyl)amine (THPTA, Sigma-Aldrich, Rehovot, IL) were added to a final concentration of 0.2 mM and 1.2 mM, respectively (1:6 ratio). 2.5 mM sodium ascorbate (Sigma-Aldrich, Rehovot, Israel) was used as a reducing agent to initiate the reaction. The reaction was incubated in RT for one hour before it was mixed with SDS sample buffer and analyzed using ExpressPlus PAGE (Genscript. Piscataway, NJ, USA). The gel was visualized in ImageQuant LAS4000 imager on a Cy3 mode (GE Healthcare, Little Chalfont, UK).

Biochemical activity assay

Biochemical activity assay of the CueO mutants was performed using o-phenylenediamine (OPD, Sigma-Aldrich, Rehovot, IL) as a substrate for CueO. Final concentrations in the reactions (200 µL total volume) were 250 µg/mL CueO mutant, 0.2 mg/mL OPD and 500 µM $CuCl_2$ in 50 mM acetate buffer pH=4, the assay was performed in triplicates in a 96-well plate and the oxidation of OPD was monitored at 436 nm in a plate reader (BioTek instruments, Winoosky, VT).

Non-specific modification of GCE

Non-specific immobilization of CueO through amine groups on the protein by 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) – N-hydroxysuccinimide (NHS) coupling with PCA. 1-Pyrenecarboxylic acid succinimide ester (PSE) was synthesized according to reference[1]. The reaction between PSE and WT CueO was done in 50 mM MES buffer pH=5.5, supplemented with 100 µM PSE (from 10 mM stock solution in DMSO) and WT CueO at 1 mg/mL, all final concentrations. The reaction mix was incubated for 1 hour at RT, and then deposited on the electrode and incubated again for 1 hour to

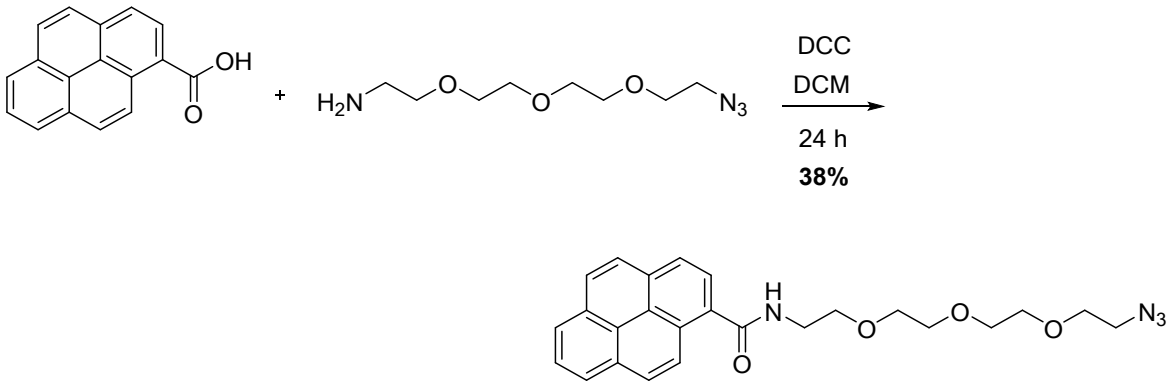
let the modified protein adsrbe to the electrode surface. After 1 hour the electrode was washed with DI water and analyzed.

General organic synthesis methods

Reactions were performed using oven-dried glassware apparatus under an atmosphere of nitrogen with anhydrous, freshly distilled solvents unless otherwise stated. All other reagents were used as obtained from commercial sources. Room temperature refers to ambient temperature. Thin-layer chromatography was performed using silica gel 60 with F254 indicator on glass plates (Merck). Flash chromatography was performed using Merck 40-63 μm silica gel. Sol-vent ratios for the purification of compounds by flash chromatography are reported as percent volume (v/v). NMR spectra were recorded using a Bruker Avance DPX400 (400 MHz) spectrometer. Spectra were calibrated on residual solvent signal. All MS analyses were performed on a Thermo Scientific LCQ Fleet mass spectrometer with an ESI source.

Synthetic procedures

N-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethyl)pyrene-1-carboxamide

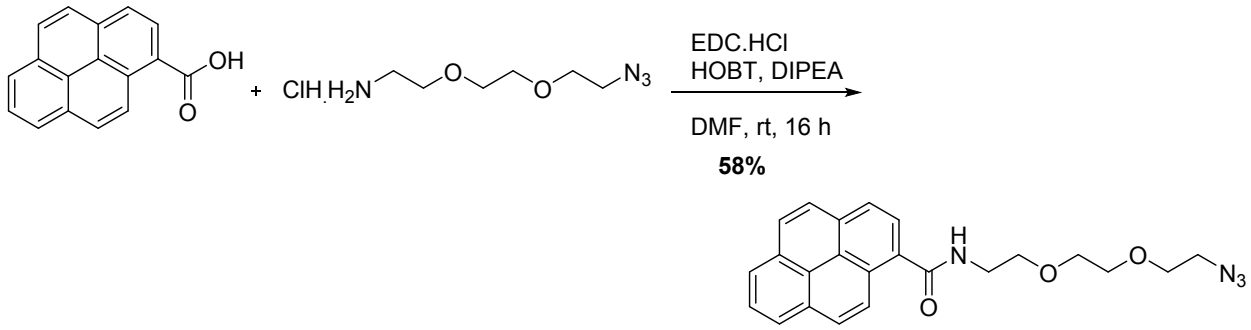


Scheme 1. Synthesis of pyrene PEG azide linker (PTAz)

A solution of DCC (167 mg, 0.81 mmol, 2 eq) in CH_2Cl_2 (5 mL) was added to a solution of the pyrene-1-carboxylic acid (100 mg, 0.40 mmol, 1 eq) and amine (106 mg, 0.48 mmol, 1.2 eq) in CH_2Cl_2 (10 mL) and the mixture was stirred at rt for 24 h. The resulting precipitate was filtered off. The solvent was evaporated and the residue chromatographed on silica gel using CH_2Cl_2 as

eluent to afford the pure product (69 mg, 38%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.60 (d, J = 9.2 Hz, 1H), 8.25 (d, J = 7.6 Hz, 1H), 8.16-8.11 (m, 4H), 8.07–8.04 (m, 2H), 6.89 (bs, 1H), 3.91–3.80 (m, 4H), 3.73-3.70 (m, 2H), 3.66-3.64 (m, 2H), 3.56–3.53 (m, 2H), 3.42–3.35 (m, 4H), 3.14 (t, J = 5.1 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) 170.14, 132.49, 131.16, 130.70, 128.62 (3C), 128.55, 127.13, 126.33, 125.80, 125.70, 124.77, 124.50, 124.40, 124.32, 70.62, 70.42, 70.28, 69.85, 69.79, 50.44, 40.67, 40.02. MS (ES mass): 447.10 (M+1).

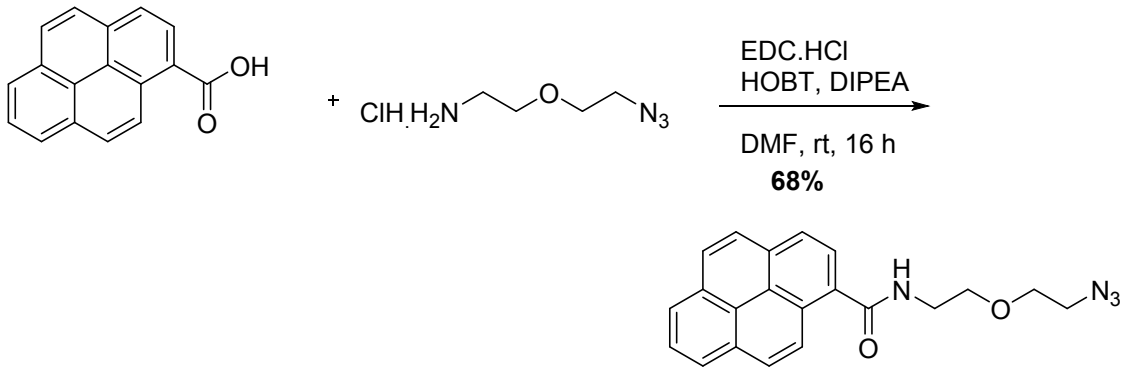
***N*-(2-(2-(2-azidoethoxy)ethoxy)ethyl)pyrene-1-carboxamide**



Scheme 2. Synthesis of pyrene PEG azide linker (PDAz)

A solution of EDC.HCl (93 mg, 0.48 mmol, 1.2 eq), HOBT (65 mg, 0.48 mmol, 1.2 eq), pyrene-1-carboxylic acid (100 mg, 0.40 mmol, 1 eq), 2-(2-(2-azidoethoxy)ethoxy)etha namine hydro chloride[2] (85 mg, 0.48 mmol, 1.2 eq), and DIPEA (0.26 mL, 1.21 mmol, 3 eq) in DMF (8 mL) was stirred at rt for 16 h. Then, the reaction mixture diluted with water (50 mL), extracted with ethyl acetate (2 x 30 mL) and the organic layer washed with brine solution (30 mL), dried over anhydrous Na_2SO_4, concentrated under reduced pressure. The residue was purified by column chromatography using ethyl acetate–hexane to give the desired compound in 58% yield (95 mg). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.59 (d, *J* = 9.2 Hz, 1H), 8.23 (d, *J* = 7.6 Hz, 1H), 8.17-8.11 (m, 4H), 8.07–8.04 (m, 2H), 6.82 (bs, 1H), 3.88–3.84 (m, 2H), 3.82-3.79 (m, 2H), 3.73-3.70 (m, 2H), 3.67–3.64 (m, 2H), 3.57 (t, *J* = 5.0 Hz, 2H), 3.20 (t, *J* = 5.0 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) NMR δ 170.24, 132.54, 131.16, 130.91, 130.68, 128.66, 128.56, 127.16, 126.34, 125.84, 125.73, 124.74, 124.44, 124.34, 70.55, 70.32, 70.02, 69.94, 50.44, 40.02. MS (ES mass): 403.1 (M+1).

***N*-(2-(2-azidoethoxy)ethyl)pyrene-1-carboxamide**



Scheme 3. Synthesis of pyrene PEG azide linker (PMAz)

2-(2-azidoethoxy)ethanamine hydrochloride was prepared according to literature procedure[3] and coupled as per above procedure in 65% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.61 (d, J = 9.2 Hz, 1H), 8.22 (d, J = 8.0 Hz, 1H), 8.16-8.10 (m, 4H), 8.07–8.02 (m, 2H), 6.70 (bs, 1H), 3.87–3.83 (m, 2H), 3.79-3.77 (m, 2H), 3.72 (t, J = 5.2 Hz, 2H), 3.39 (t, J = 5.0 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.08, 132.55, 131.14, 130.80, 130.68, 128.64, 127.15, 126.31, 125.81, 125.73, 124.70, 124.42, 124.35, 70.10, 70.00, 50.68, 39.95. MS (ES mass): 359.1 (M+1).

References:

(1) Saito, I.; Saito, Y.; Matsumoto, Y. JP2010060394A 2008.

(2) Liu, X.; Zhou, J.; Yu, T.; Chen, C.; Cheng, Q.; Sengupta, K.; Huang, Y.; Li, H.; Liu, C.; Wang, Y.; Posocco, P.; Wang, M.; Cui, Q.; Giorgio, S.; Fermeglia, M.; Qu, F.; Pricl, S.; Shi, Y.; Liang, Z.; Rocchi, P.; Rossi, J. J.; Peng, L. *Angew. Chemie Int. Ed.* **2014**, *53* (44), 11822–11827.

(3) Bogdan, A. R.; Davies, N. L.; James, K. *Org. Biomol. Chem.* **2011**, *9* (22), 7727.

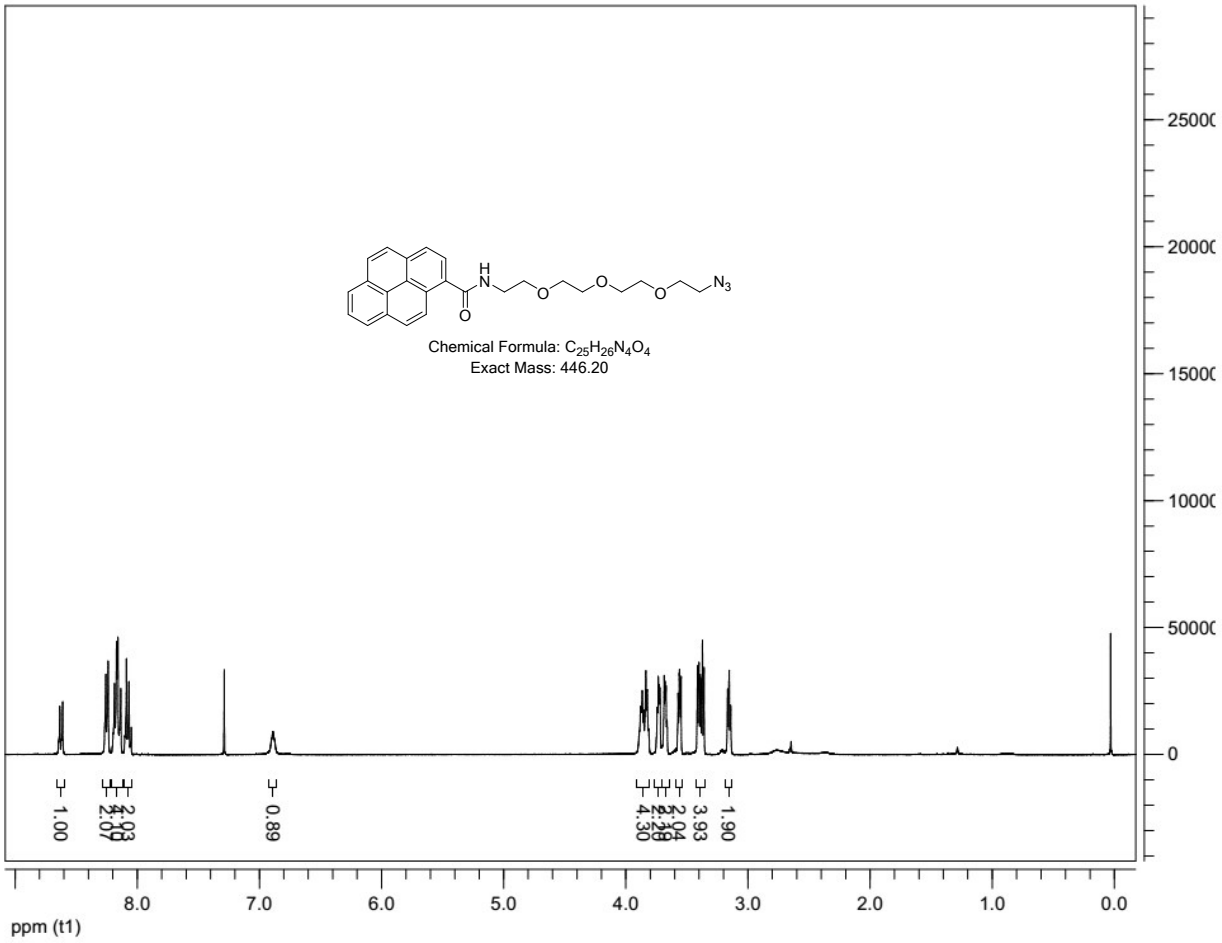


Figure S4. ^{1}H NMR of pyrene PEG azide linker (PTAz)

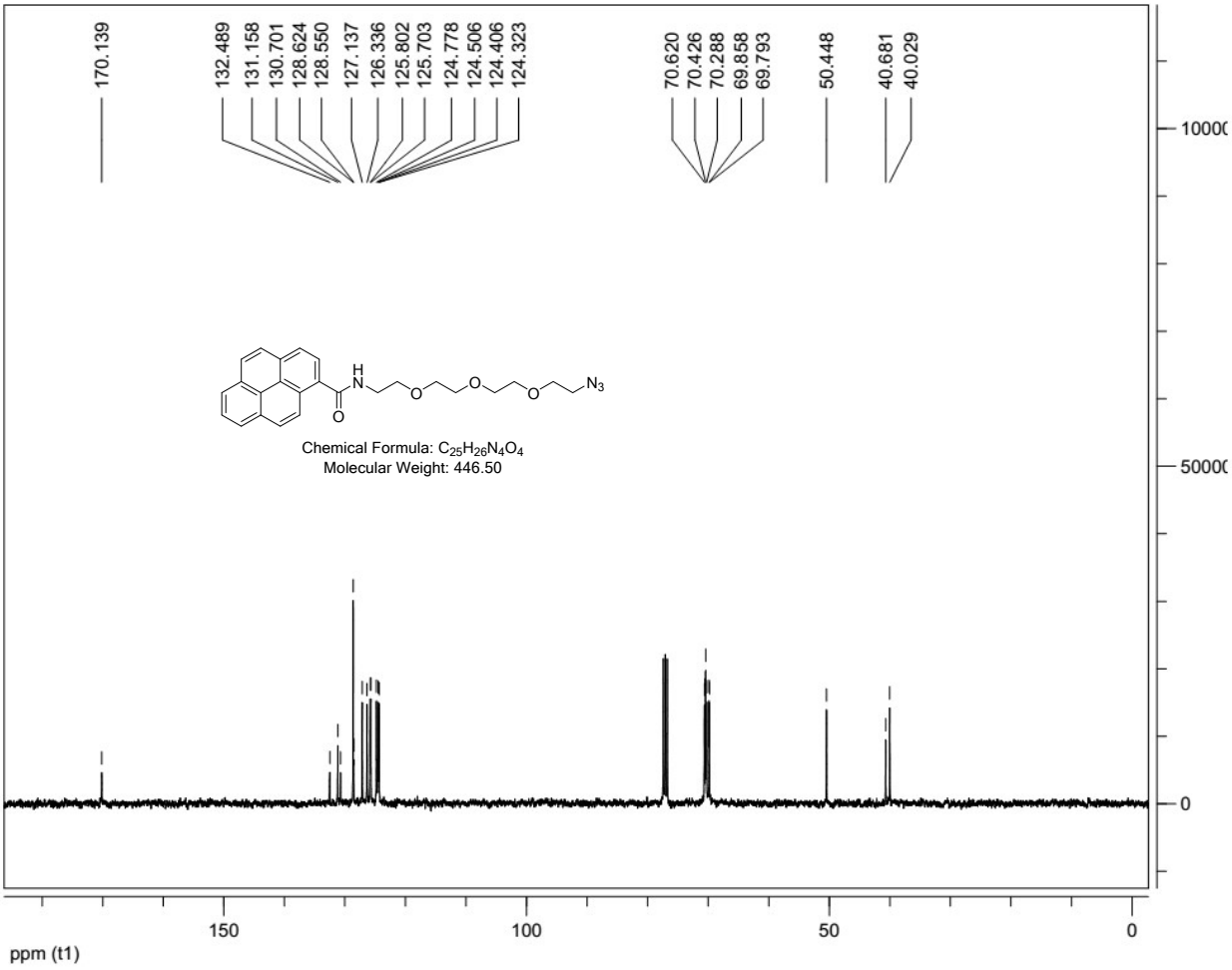


Figure S5. ^{13}C NMR of pyrene PEG azide linker (PTAz)

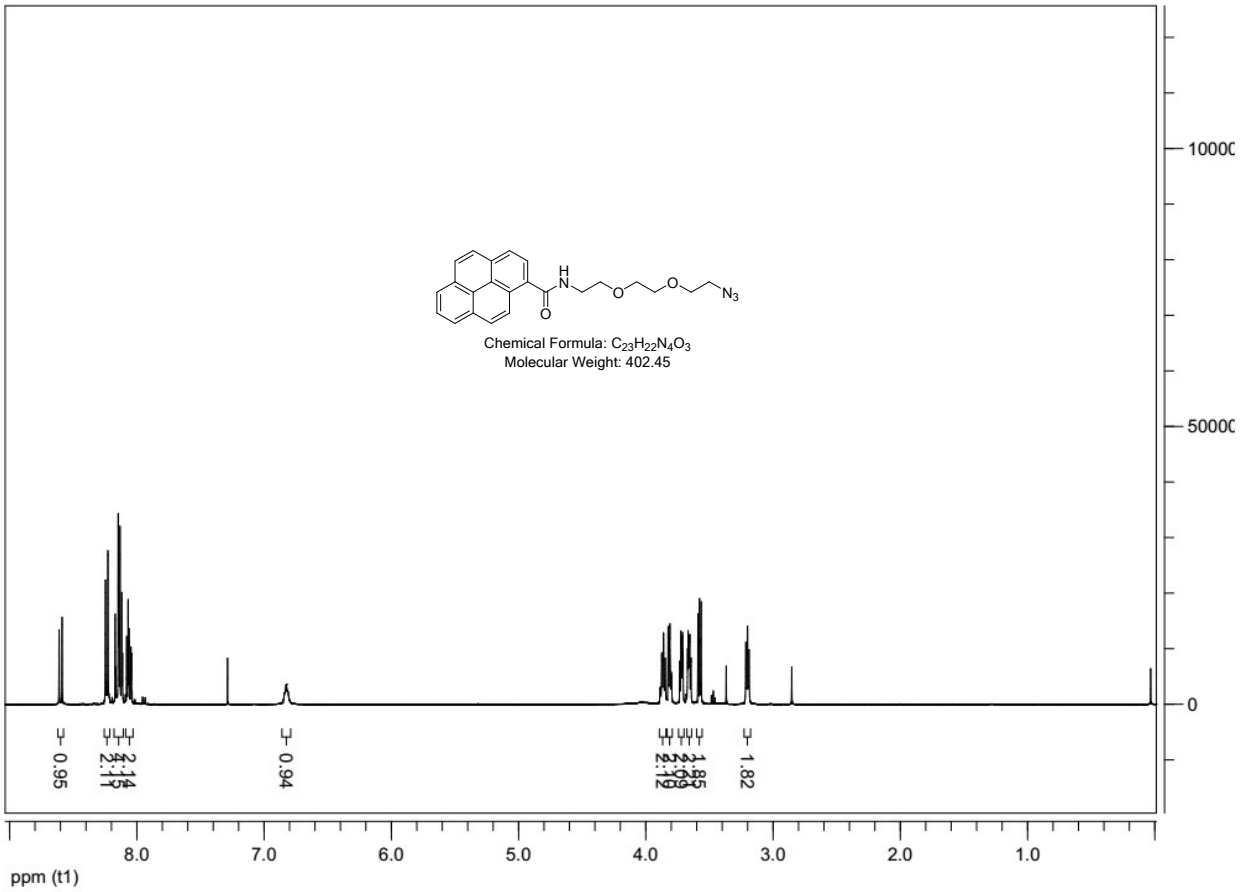


Figure S6. ^{1}H NMR of pyrene PEG azide linker (PDAz)

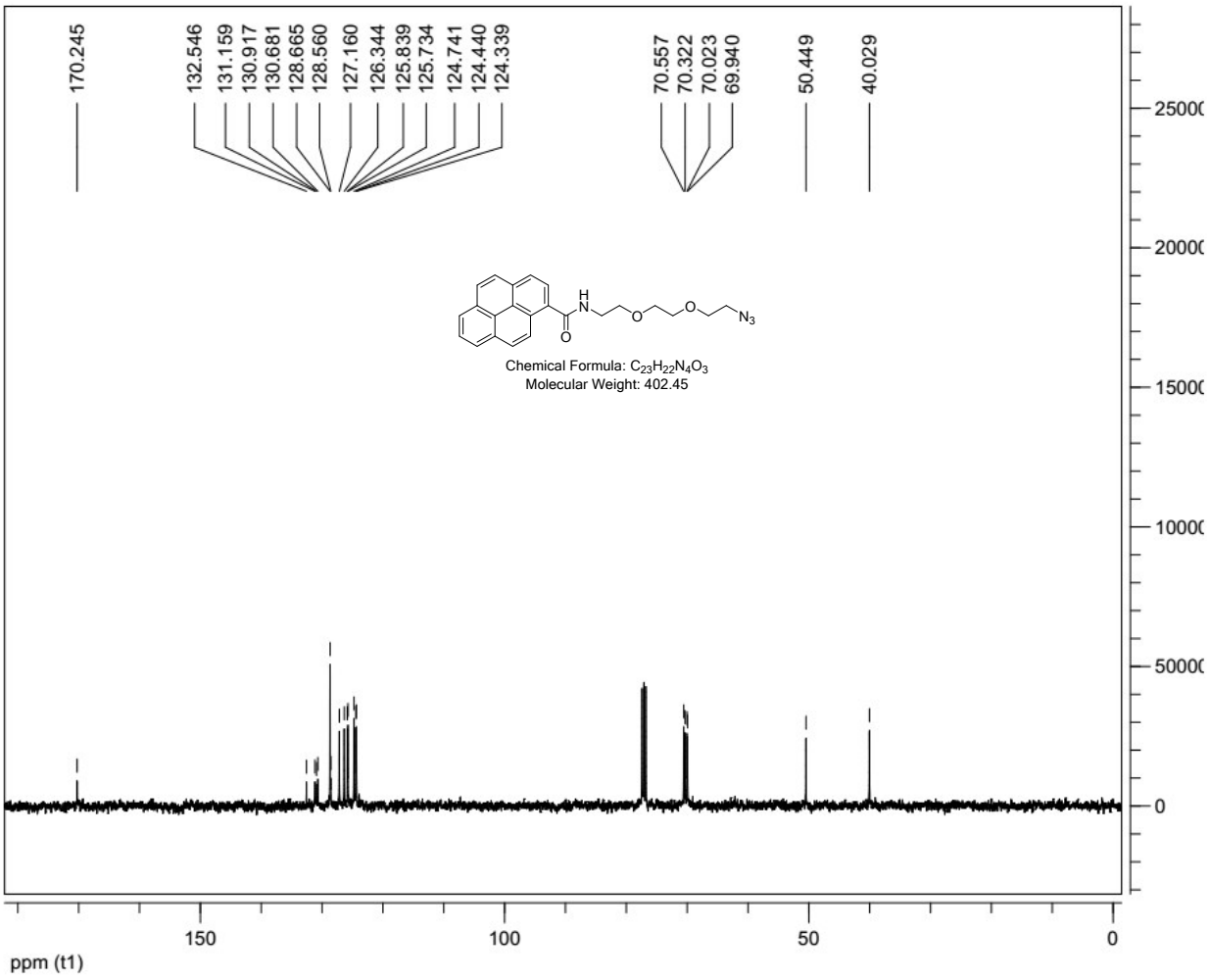


Figure S7. ^{13}C NMR of pyrene PEG azide linker (PDAz)

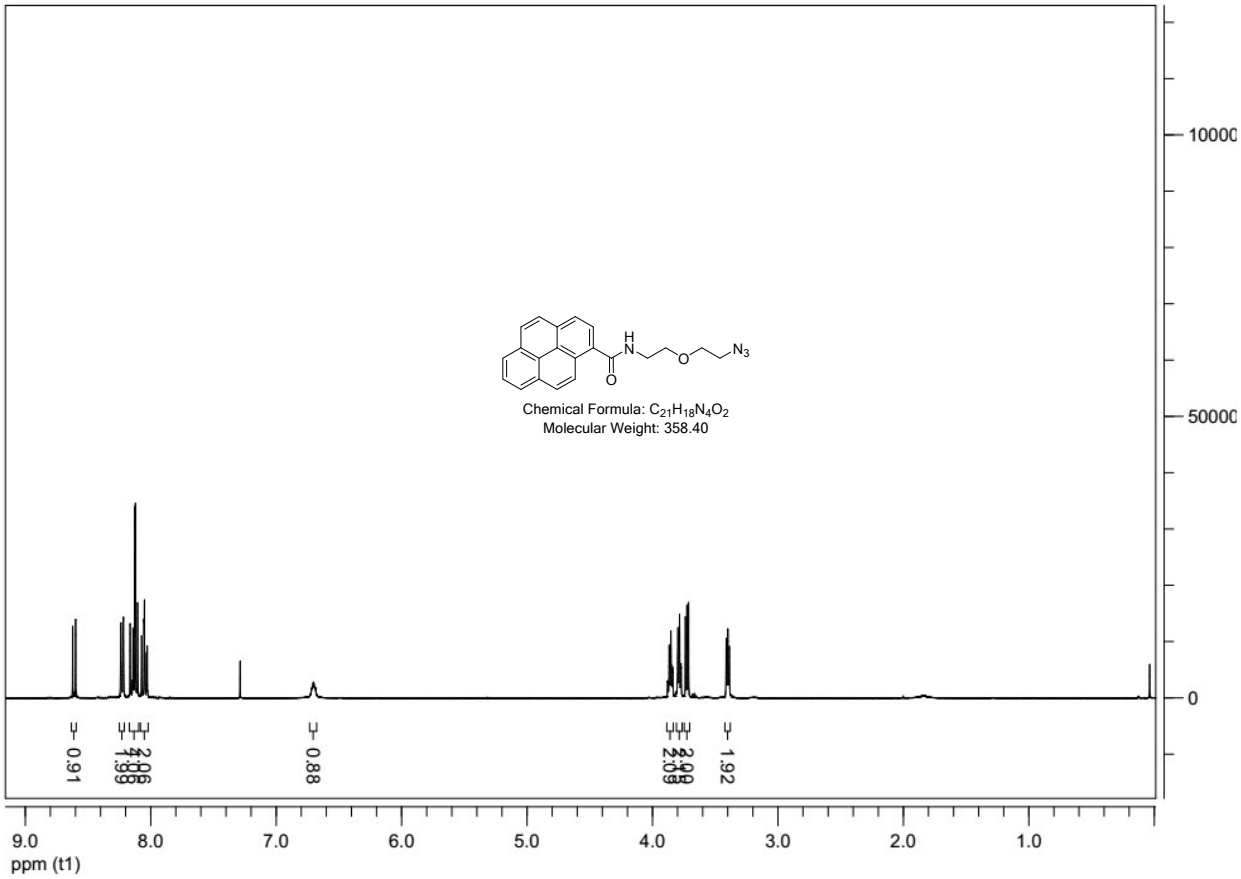


Figure S8. ^{1}H NMR of pyrene PEG azide linker (PMAz)

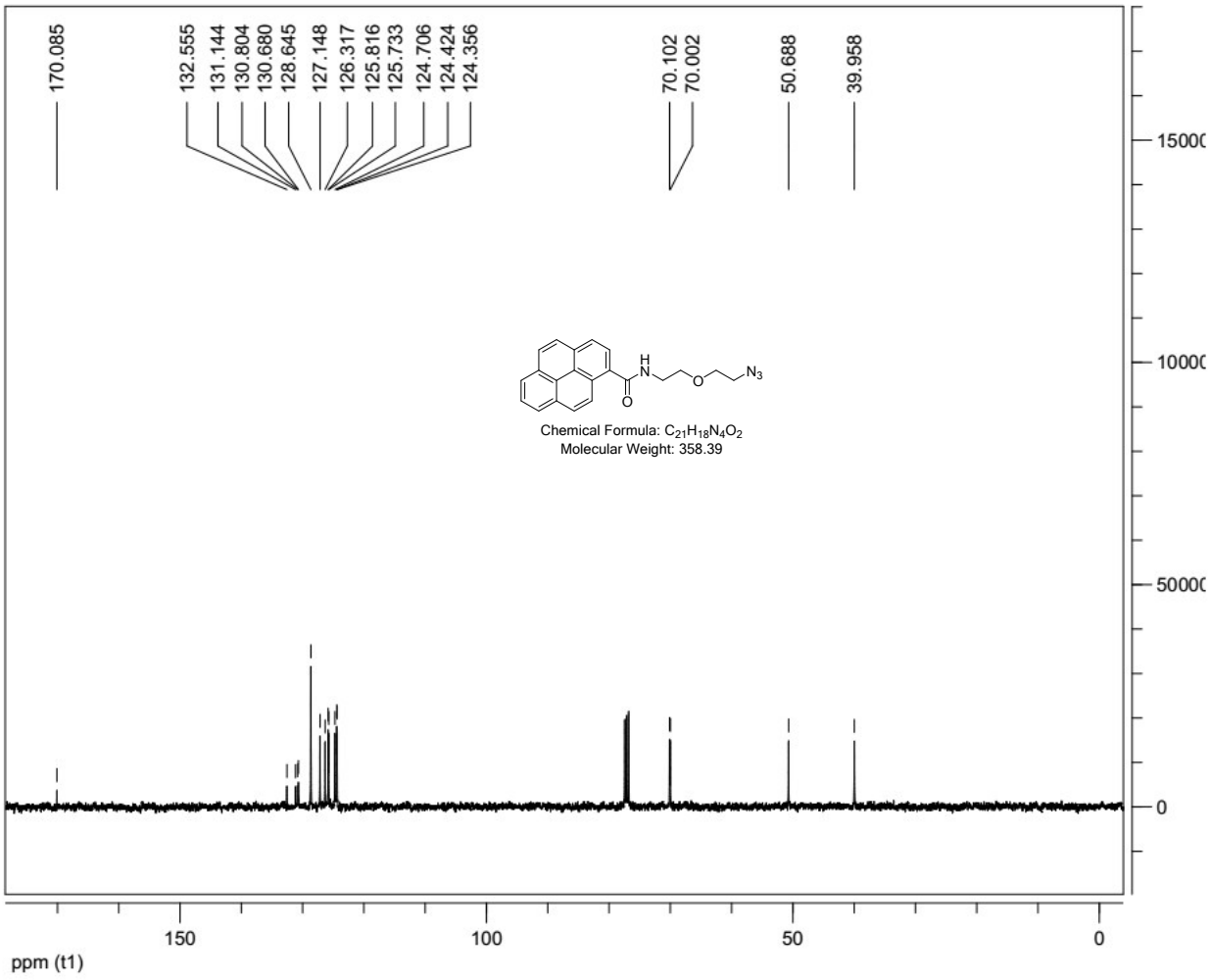


Figure S9. ^{13}C NMR of pyrene PEG azide linker (PMAz)