

Copper-catalysed cycloaddition reactions of nitrones and alkynes for bioorthogonal labelling of living cells

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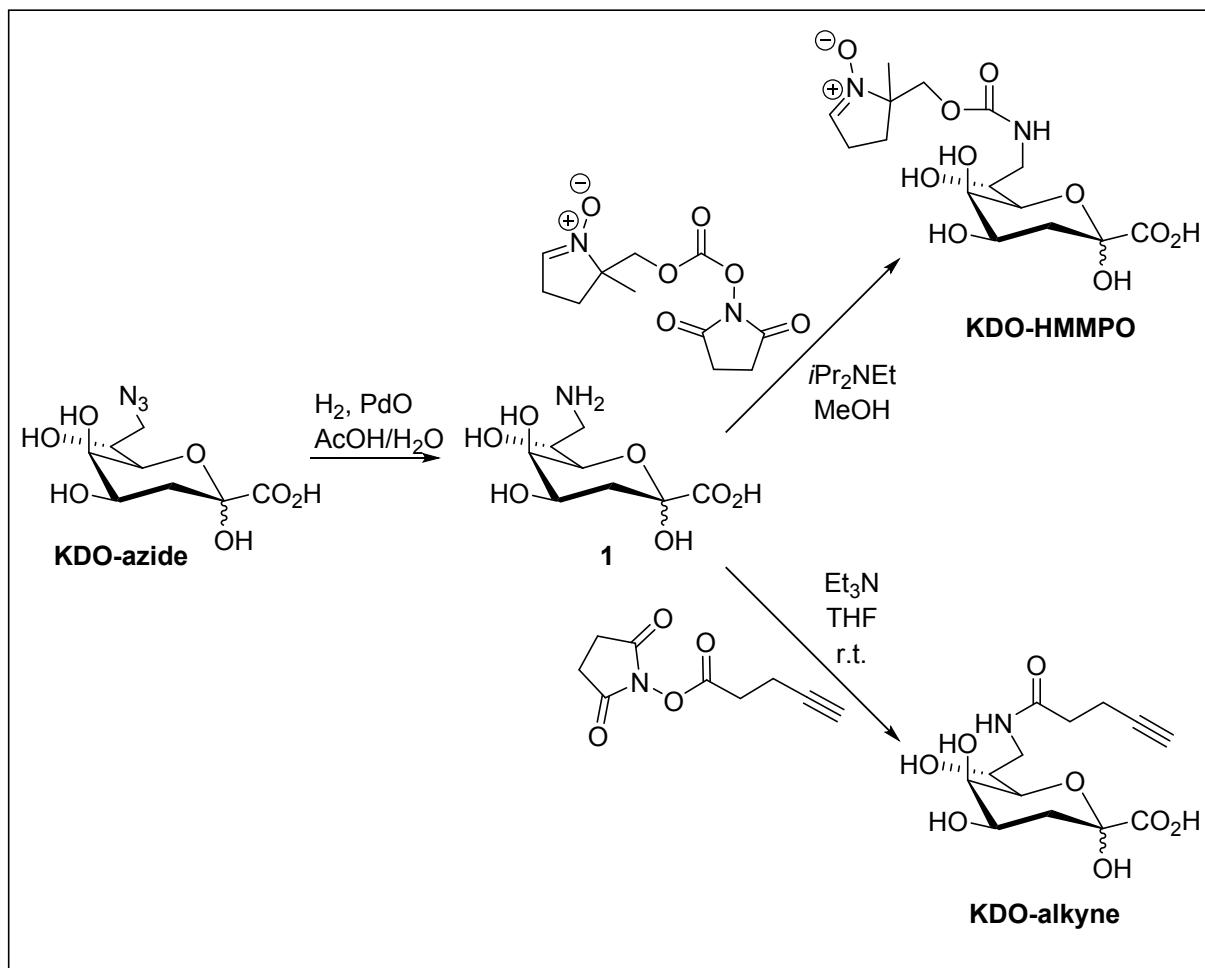
Materials and Synthetic Methods

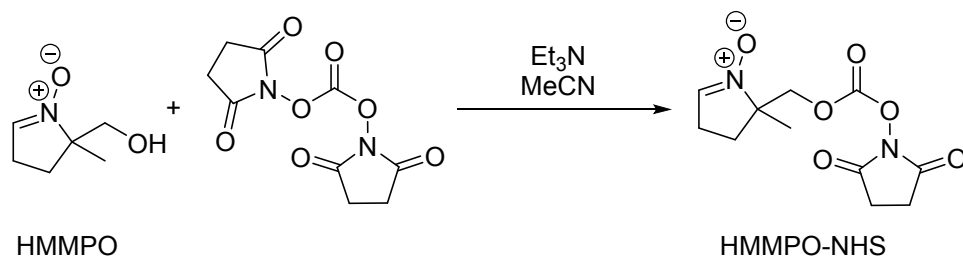
All reagents and solvents were purchased from Sigma-Aldrich, unless otherwise stated, and used without further purification. Deuterated solvents were purchased from Cambridge Isotope laboratories. Thin layer chromatography was performed on Analtech Uniplate® silica gel plates (60 Å F254, layer thickness 250µm). Flash chromatography was performed using silica gel (60 Å, particle size 40–63 µm). LC-MS/MS spectra were obtained using Waters Alliance 2795 liquid chromatograph equipped with Waters 996 PDA diode array detector and connected to Micromass ZQ2000 mass spectrometer equipped with pneumatically assisted electrospray ionization source, operating in both positive and negative mode. Samples were run with gradient elution of acetonitrile/water/0.1% formic acid on the Waters SunFire C18 (2.1 x 100 mm, 3.5 µm) column with flow rate of 0.2 mL/min. Preparatory HPLC was performed on a Waters Delta Prep 4000 equipped with Waters 996 PDA diode array detector and column Waters SunFire C18 (19 x 100 mm, 5 µm) and Waters fraction collector. Column effluents were monitored at wavelength specified in respective procedures. Mobile phase was acetonitrile/water/0.1% formic acid, degassed with helium sparge at flow rate of 17 mL/min. Aliquots were injected onto column via a Pheodyne 7125 injector fitted with a 5 mL loop. All ¹H and ¹³C NMR spectra were obtained on a Bruker-DRX-400 spectrometer using a frequency of 400 MHz for ¹H and 100 MHz for ¹³C and processed using Bruker TOPSPIN 2.1 software. The following abbreviations were used to designate chemical shift multiplicities: s = singlet, d = doublet, t = triplet, m = multiplet or unresolved, br = broad signal and *J* = coupling constants in Hz.

Synthesis of sugar derivatives

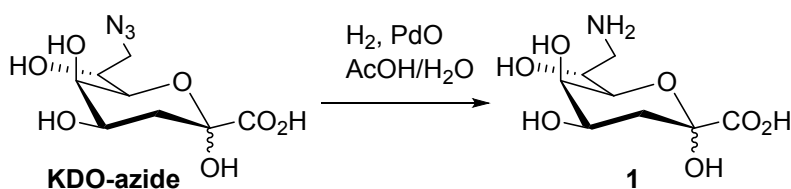
Ac₄ManN-alkyne was synthesized according to the previously reported procedure.¹

Synthesis of KDO-HMMPO and KDO-alkyne from KDO-azide

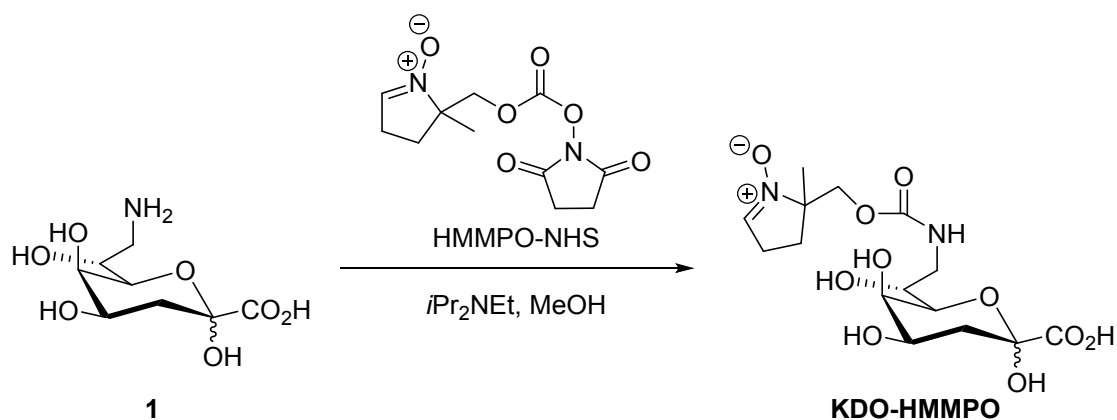




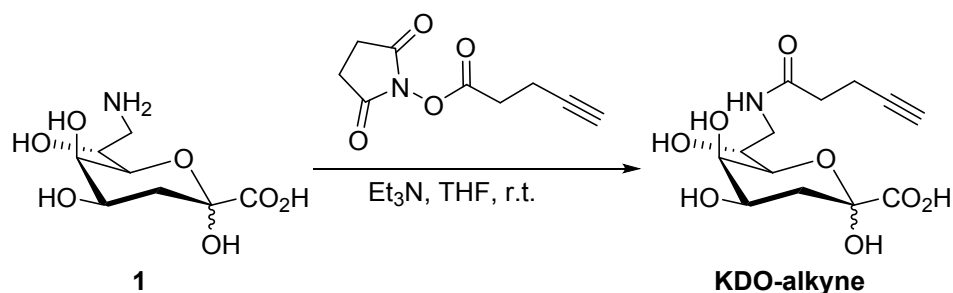
HMMPO synthesis and characterization data has been previously reported in literature.² To a solution of HMMPO (621 mg, 4.81 mmol, 1 eq) and disuccinimide carbonate (1.48 g, 5.77 mmol, 1.2 eq) in dry acetonitrile (50 mL) under argon atmosphere was added triethylamine (0.87 mL, 6.25 mmol, 1.3 eq). The mixture was stirred at room temperature overnight. Upon confirmation of product formation by MS, the mixture was concentrated and the crude was used without further purification. **MS (ESI+)** calcd ($\text{C}_{11}\text{H}_{14}\text{N}_2\text{O}_6$): 270.09 $[\text{M}+\text{H}]^+$, found 271.2.



KDO-azide was synthesized following procedures previously reported in literature.³ To a solution of KDO-azide (433.0 mg, 1.65 mmol, 1.0 eq) in water (16.7 mL) and acetic acid (20 drops) was added palladium oxide (109.0 mg, 0.89 mmol, 0.54 eq). Hydrogen gas was bubbled through the reaction and the solution was stirred under hydrogen gas over 23 hours. Solid was filtered off over Celite under vacuum, the cake rinsed once with water. Filtrate was concentrated and further dried under high vacuum to yield crude product **1** as yellow solid. ($\text{H}_2\text{O}:\text{iPrOH}/1:9$, $R_f = 0.25$). Crude **1** was immediately used in the next reaction without further purification. **MS (ESI+)** calcd ($\text{C}_8\text{H}_{15}\text{NO}_7$): 237.08 $[\text{M}+\text{H}]^+$, found 238.2.

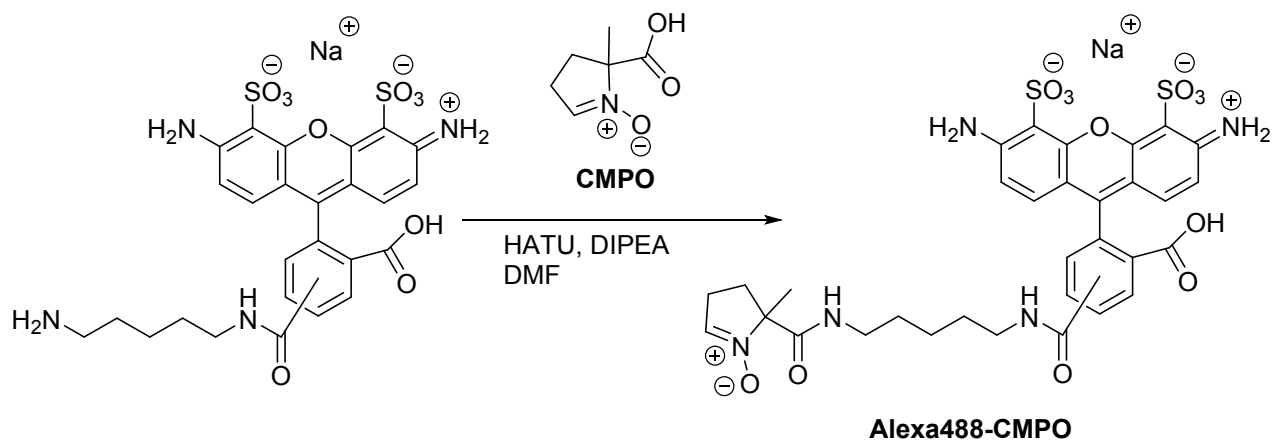


To a suspension of **1** in methanol was added $i\text{Pr}_2\text{NEt}$ until basic pH was achieved. HMMPO-NHS was added drop wise over 5 minutes at room temperature. Reaction was stirred at r.t. for 4 hours. The yellow suspension was concentrated and stored under argon at -78°C . The mixture was purified by preparatory HPLC with MeCN/ H_2O /FA (0.1%) as eluent, using isocratic method with 2% MeCN for 3.5 minutes, then 5% MeCN for 4.5 minutes. Product eluted at ~ 6 minutes and its presence was confirmed by MS; the fractions were pooled and concentrated under reduced pressure. Product was obtained as off-white solid (62.0 mg, 16.5%). **HRMS** m/z calcd ($\text{C}_{15}\text{H}_{24}\text{N}_2\text{O}_{10}$) $[\text{M}+\text{Na}]^+$: 415.13293, found 415.13232; **$^1\text{H-NMR}$** (400 MHz, D_2O) δ 8.21(s, 2H), 7.30 (s, 1H), 4.47 (m, 1H), 4.24 (m, 1H), 4.14 (m, 1H), 3.96 (m, 3H), 3.70 (m, 2H), 3.45 (m, 2H), 3.19 (m, 1H), 2.69 (m, 3H), 2.29 (m, 3H), 1.99 (m, 1H), 1.37 (s, 3H); **$^{13}\text{C-NMR}$** (100 MHz, D_2O) δ 175.6, 173.4, 165.8, 158.0, 145.1, 95.4, 76.1, 72.6, 67.6, 66.4, 66.0, 65.6, 52.3, 43.7, 33.0, 28.7, 28.2, 25.7, 19.9.



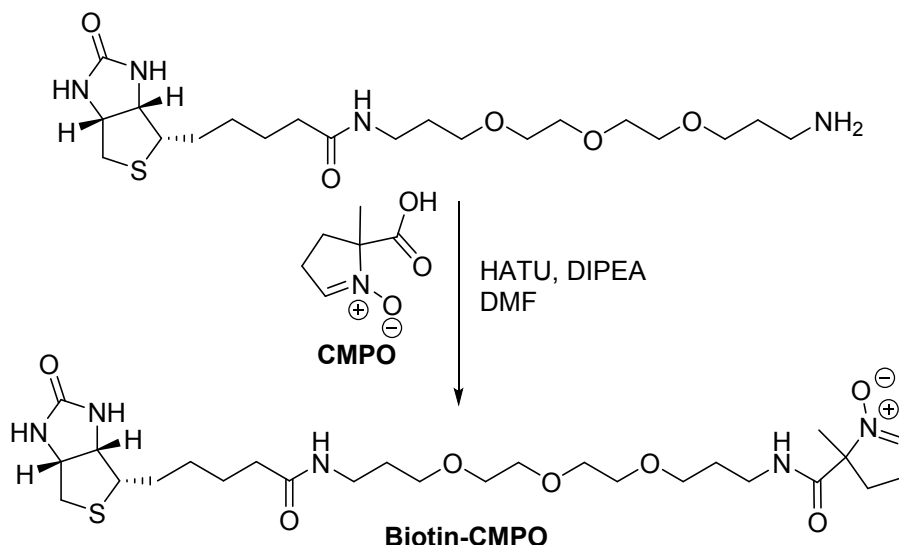
1 (crude, 1.6 mmol) was dissolved in THF (8.24 mL), NHS-alkyne⁴ (320.0 mg, 1.65 mmol, 1.0 eq) and triethylamine (2.76 mL, 19.8 mmol, 12.0 eq) were added and the solution was stirred at room temperature under argon for 43 hours. The solution was concentrated under reduced pressure to a yellow solid, which was stored under argon at -78°C. The crude was purified by preparatory HPLC with MeCN/H₂O/FA (0.1%) as eluent, running gradient of 5 to 25% acetonitrile over 10 minutes. The presence of product in eluted fractions was confirmed by the MS; the fractions were pooled and concentrated under reduced pressure. The product was obtained as red foam (71.0 mg, 13.7%). **MS (ESI-)** calcd (C₁₃H₁₉NO₈): 316.10 [M-H]⁻, found 316.1; **¹H-NMR** (400 MHz, D₂O) δ 4.08 (m, 2H), 3.94 (m, 1H), 3.80 (d, 1H, *J*=9.1 Hz), 3.60 (m, 1H), 3.30 (m, 1H), 2.79 (s, 3H), 2.50 (m, 3H), 2.37 (s, 1.5H), 1.98 (m, 2H); **¹³C-NMR** (100 MHz, D₂O) δ 176.4, 83.3, 72.6, 70.1, 69.9, 67.4, 65.9, 65.6, 48.8, 42.5, 34.4, 32.9, 25.1, 14.5, 14.2.

Synthesis of Alexa488-CMPO



Alexa Fluor® 488 Cadaverine was purchased from Life Technologies, care was taken to protect the reagent and the reaction mixture from light. Nitron (CMPO) was synthesized according to previously reported procedure.⁵ Alexa Fluor® (1 mg, 0.0016 mmol, 1 eq), CMPO (1 mg, 0.007 mmol, 4.4 eq) and HATU (1 mg, 0.0026 mmol, 1.6 eq) were dissolved in DMF (10 uL). DIPEA (0.5 uL, 0.003 mmol, 1.76 eq) was added all at once and the mixture was stirred overnight, protected from light. Formation of product was confirmed by LC-MS. The crude was diluted with water (1 mL) and purified using preparatory HPLC with MeCN/H₂O/FA (0.1%) as eluent, running gradient of 10 to 95% acetonitrile over 20 minutes and monitoring at 220 nm and 498 nm. Product eluted at 6-8 minutes and its presence was confirmed by MS; the fractions were pooled and concentrated under reduced pressure. The product was obtained as orange-red solid (0.59 mg, 0.0008 mmol, 50 % yield), stored in freezer, protected from light. **MS (ESI+)** calcd (C₃₂H₃₂N₅O₁₂S₂⁻): 742.15 [M]⁻, found 742.4, 744.15 [M+2H]⁺, found 744.3.

Synthesis of Biotin-CMPO



The Biotin-PEG⁶ and nitron (CMPO)⁵ were synthesized according to previously reported procedures. Biotin-PEG (91 mg, 0.20 mmol, 1 eq), CMPO (35 mg, 0.24 mmol, 1.2 eq) and HATU (76 mg, 0.20 mmol, 1 eq) were dissolved in DMF (175 μ L). DIPEA (52 μ L, 0.30 mmol, 1.75 eq) was added all at once and the mixture was stirred for 45 minutes. Reaction progress was confirmed by LC-MS. The reaction was concentrated under reduced pressure and stored at -20°C overnight. The crude was purified using preparatory HPLC with MeCN/H₂O/FA (0.1%) as eluent, running gradient of 10 to 60% acetonitrile over 15 minutes. The product eluted at 7.5-8 minutes and its presence was confirmed by MS; the fractions were pooled and concentrated under reduced pressure. Some starting material was also isolated. The product was obtained as colourless oil (20.1 mg, 0.035 mmol, 17.5 % yield). **MS (ESI+)** calcd (C₂₆H₄₅N₅O₇S): 572.30 [M+H]⁺, found 572.1; **¹H-NMR** (400 MHz, MeOD) δ 7.24 (s, 1H), 4.52 (dd, 1H, $J=4.9, 7.7$ Hz), 4.33 (dd, 1H, $J=4.4, 7.8$ Hz), 3.64 (m, 9H), 3.55 (dt, 4H, $J=4.7, 6.0, 6.1$ Hz), 3.36 (m, 2H), 3.25 (m, 3H), 2.95 (dd, 1H, $J=4.9, 12.7$ Hz), 2.71 (dd, 3H, $J=10.6, 14.1$ Hz), 2.22 (t, 3H, $J=7.4, 7.4$ Hz), 1.79 (ddd, 5H, $J=4.0, 6.4, 12.8$ Hz), 1.69 (s, 3H), 1.62 (m, 3H), 1.47 (dd, 2H, $J=7.5, 15.2$ Hz).

Hz); **¹³C-NMR** (100 MHz, MeOD) δ 174.6, 170.8, 141.1, 79.1, 70.1, 69.9, 69.8, 68.6, 68.5, 62.0, 60.2, 55.6, 39.7, 37.0, 36.4, 35.5, 31.0, 29.0, 28.8, 28.4, 28.1, 25.5, 25.0, 21.5.

Experimental Methods

Fluorescence microscopy. All microscopy images herein were acquired using an Olympus 1 x 81 spinning-disk confocal microscope equipped with a Photometrics (Coolsnap ES) camera and FITC filter (Semrock, Excitation: 465-499 nm, Emission: 516-556 nm). Images were acquired using either 60X or 100X magnification using both bright field and FITC channel (4s exposure). Images were processed using ImageJ software to apply pseudocolouring to the FITC channel, and to apply the same pixel-intensity ranges for samples and paired controls.

CuANCR labelling of alkyne magnetic beads. Approximately 1.5×10^7 alkyne magnetic beads (Click Chemistry Tools) per sample were washed three times in PBS, then incubated with 50 μ M CMPO-functionalized reporter in PBS alone, or PBS containing 0.1 mM CuSO₄, 0.2 mM L-Histidine, 2 mM sodium ascorbate for 30 minutes at 37°C. Beads were subsequently washed three times in PBS and imaged directly (for Alexa488-CMPO treated), or stained with 5 μ g/ml FITC-streptavidin (for Biotin-CMPO treated) for 30 minutes at room temperature, washed in PBS, then imaged.

Metabolic labelling of Huh-7 cells via CuANCR. Huh-7 cells were cultured on cover slips in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin, 50 mg/ml streptomycin, and 100 nM nonessential amino acids, in the absence or presence of 50 μ M Ac₄ManN-alkyne for 72 hours. Cells were then washed 3 times in PBS and reacted with 50 μ M Biotin-CMPO in PBS containing 0.1 mM CuSO₄, 0.2 mM L-Histidine, 2 mM sodium ascorbate for 30 minutes at 37°C, washed 3 times in PBS, blocked in 1% BSA (in PBS) for 20 minutes at room temperature, and then stained with 5 μ g/ml FITC-streptavidin (in PBS) for 30 minutes at room temperature. Cells were then washed 3 times in

PBS, and fixed for 15 minutes at 4°C in 4% paraformaldehyde and 4% sucrose in PBS before imaging.

Metabolic labelling of *E. coli* cells via CuANCR. BL21 *E. coli* were inoculated into minimal M9 medium containing 4 mM KDO or functionalized KDO derivative and cultured at 37°C for 16 hours prior to washing 3 times in PBS. Cells were treated with 50 µM reporter (alkyne or nitrene) in PBS containing 0.1 mM CuSO₄, 0.2 mM L-Histidine, 2 mM sodium ascorbate for 30 minutes at 37°C then washed in PBS 3 times, prior to imaging of live cells by fluorescence microscopy.

LPS extraction, imaging and silver staining. BL21 *E. coli* cultured in 4 mM KDO or functionalized KDO derivative were labelled, as above, in pre-weighed 1.5ml microfuge tubes. After the last wash, bacterial pellets were weighted and resuspended in SDS-PAGE Laemmli buffer (10% SDS, 50% Glycerol in 0.5M Tris-Cl pH 6.8) at a 1mg/30µl concentration. The lysates were incubated at 100°C for 10 minutes, and then cooled to room temperature before an equal volume of SDS-PAGE Laemmli buffer was added with 100ug of Proteinase K (Life Technologies, Burlington, ON). After incubation for one hour at 60°C, 30µl samples containing 500µg of cell mass were run on a 15% SDS-PAGE gel. The gel was stopped when the dye front had migrated 6-7 cm, and then immediately visualized using the ChemiDoc™ MP (Bio-Rad, Mississauga, ON) with the Blue Epi illumination source and the 530/28 excitation filter. The gel was silver stained as previously described.⁷

Supplementary Figures

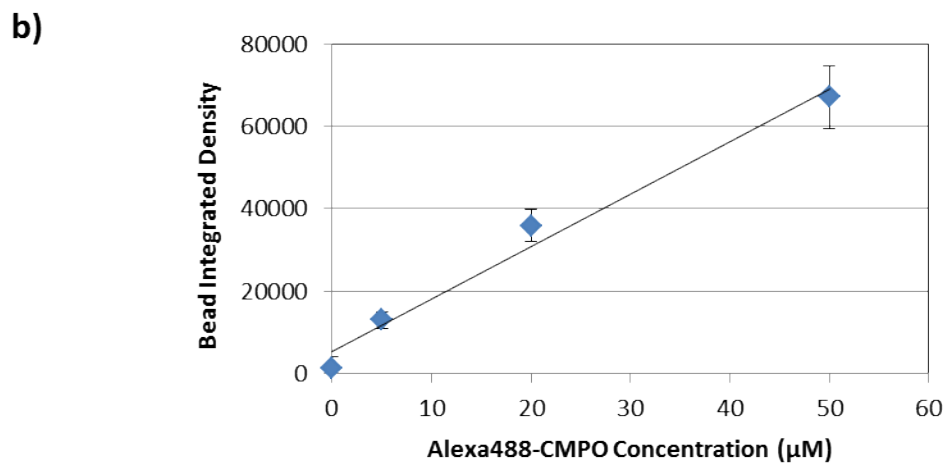
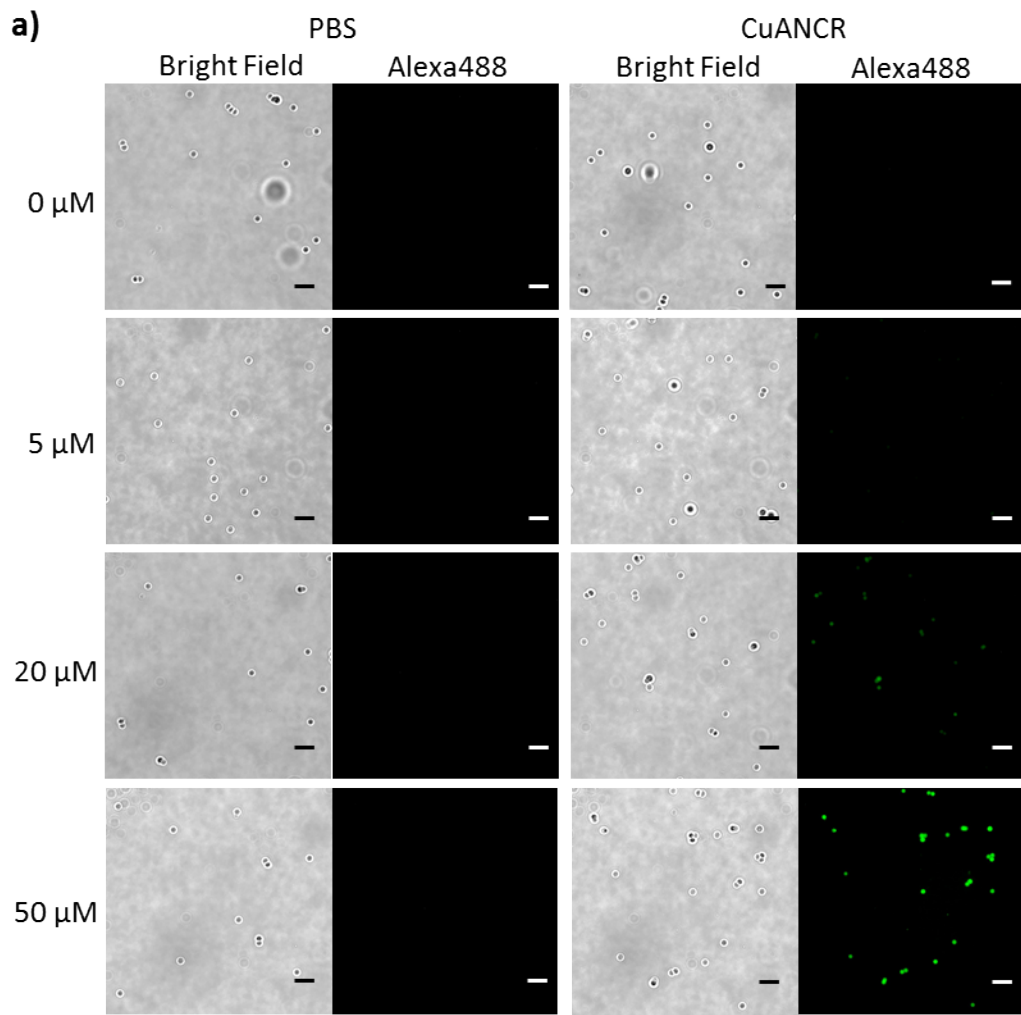
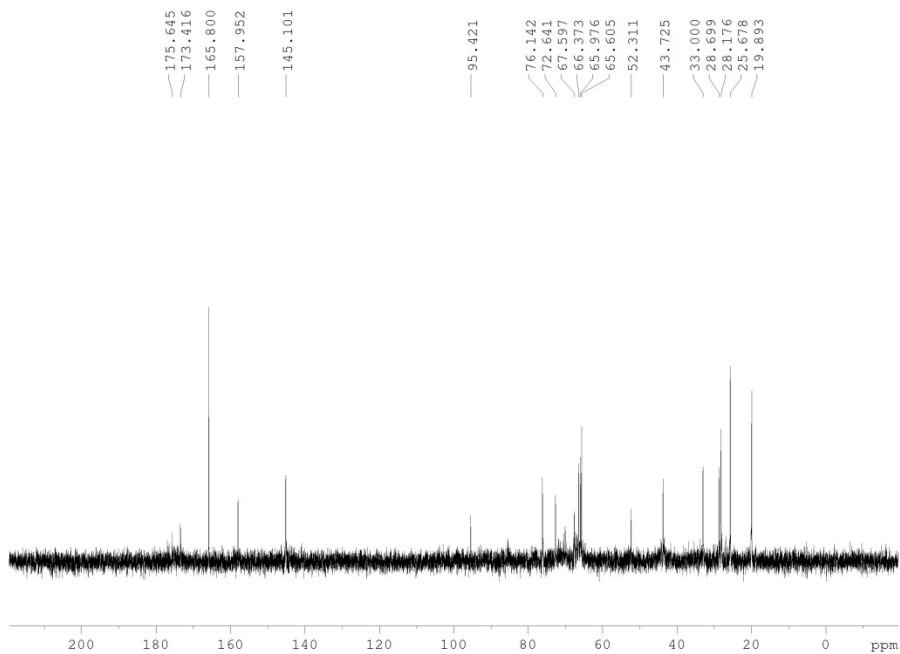
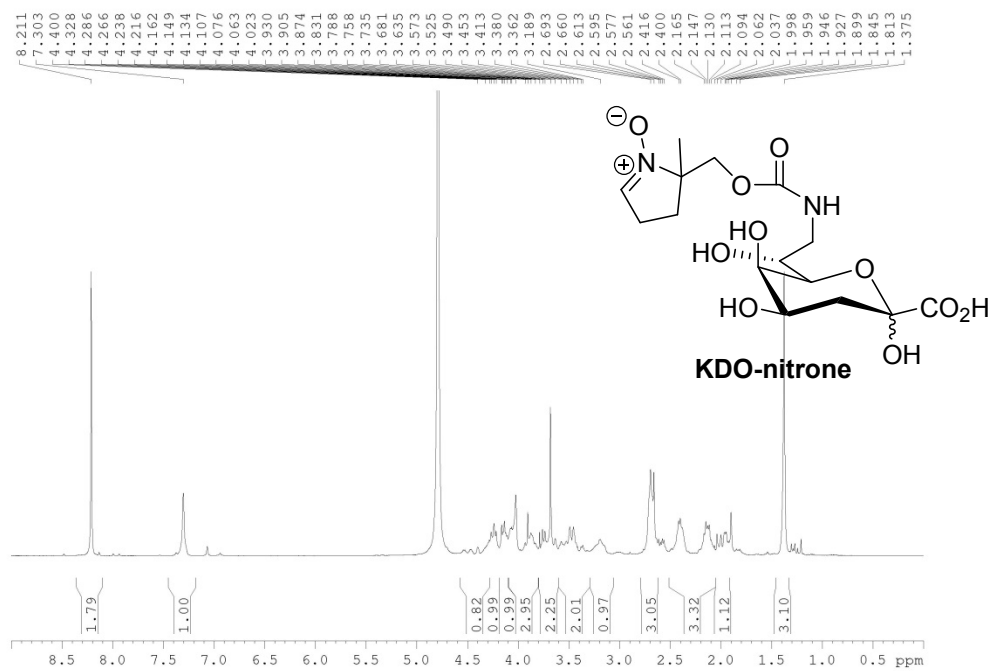
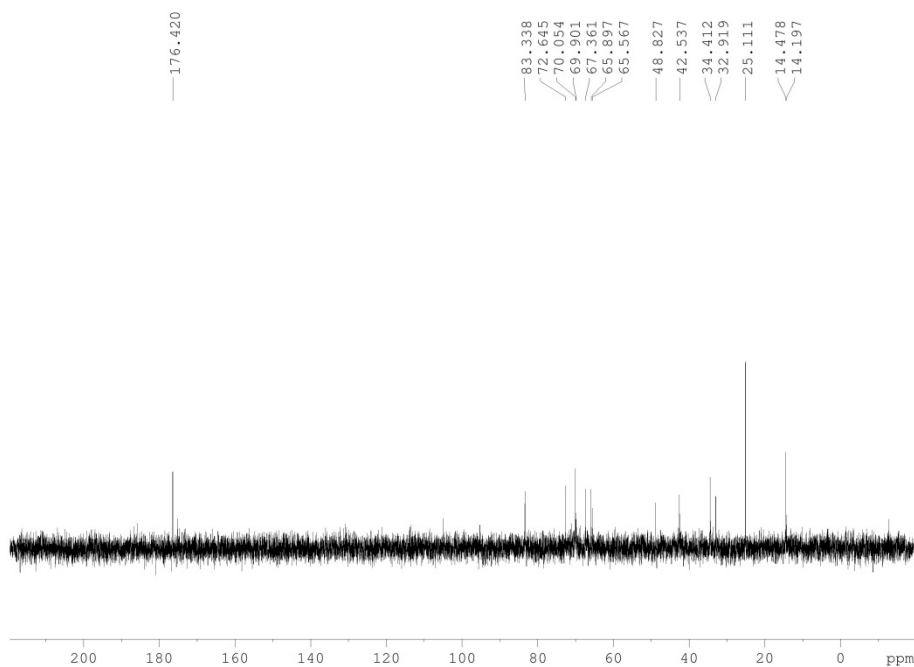
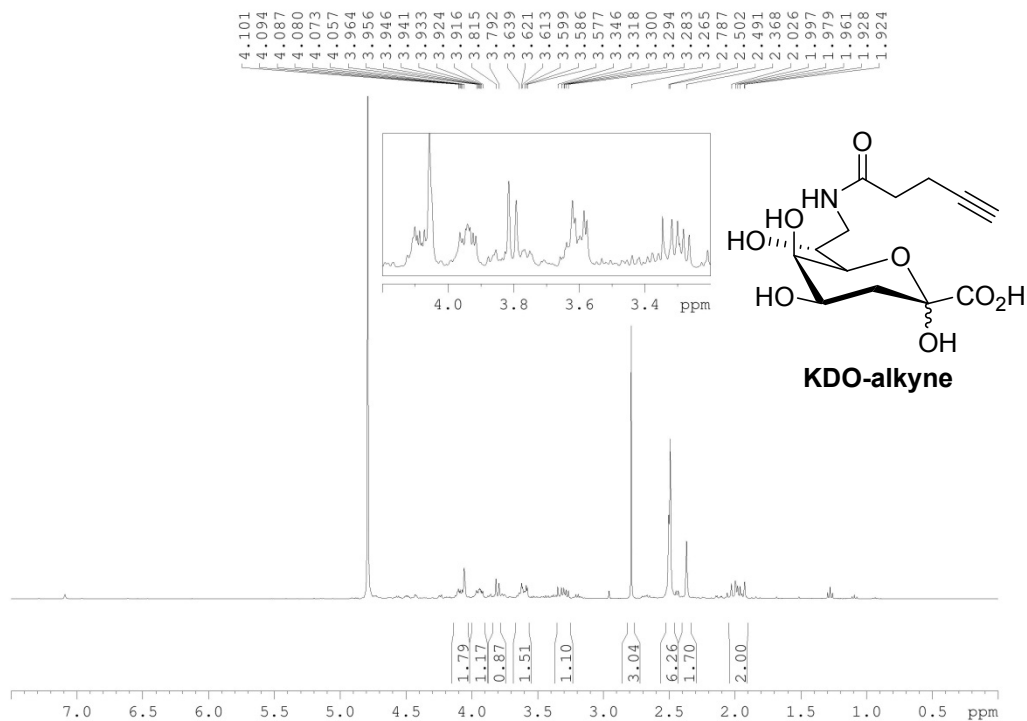


Fig. S1: Dose-dependent labelling of alkyne-tagged magnetic beads with Alexa488-CMPO by CuANCR. (a) Fluorescence microscopy of Alexa488-CMPO labelled alkyne beads. Alkyne beads were incubated in the presence of various concentrations of Alexa488-CMPO in PBS alone ("PBS"), or PBS containing 0.1 mM CuSO₄, 0.2 mM L-Histidine, 2 mM sodium ascorbate ("CuANCR") for 30 minutes at 37°C. Beads were washed in PBS then imaged by fluorescence microscopy. All fluorescence images were captured with identical exposure time, and adjusted to eliminate background fluorescence in control samples. (b) Fluorescence intensity of alkyne beads labelled via CuANCR is concentration dependent. All acquired images were adjusted to have identical minimum and maximum brightness levels, and bead fluorescence was measured using the circle selection tool to determine integrated density of 5 beads per image. The data points represent the average labelling (CuANCR conditions) minus background (Alexa488-CMPO in PBS) for each concentration of Alexa488-CMPO. Scale bars = 5 μm.

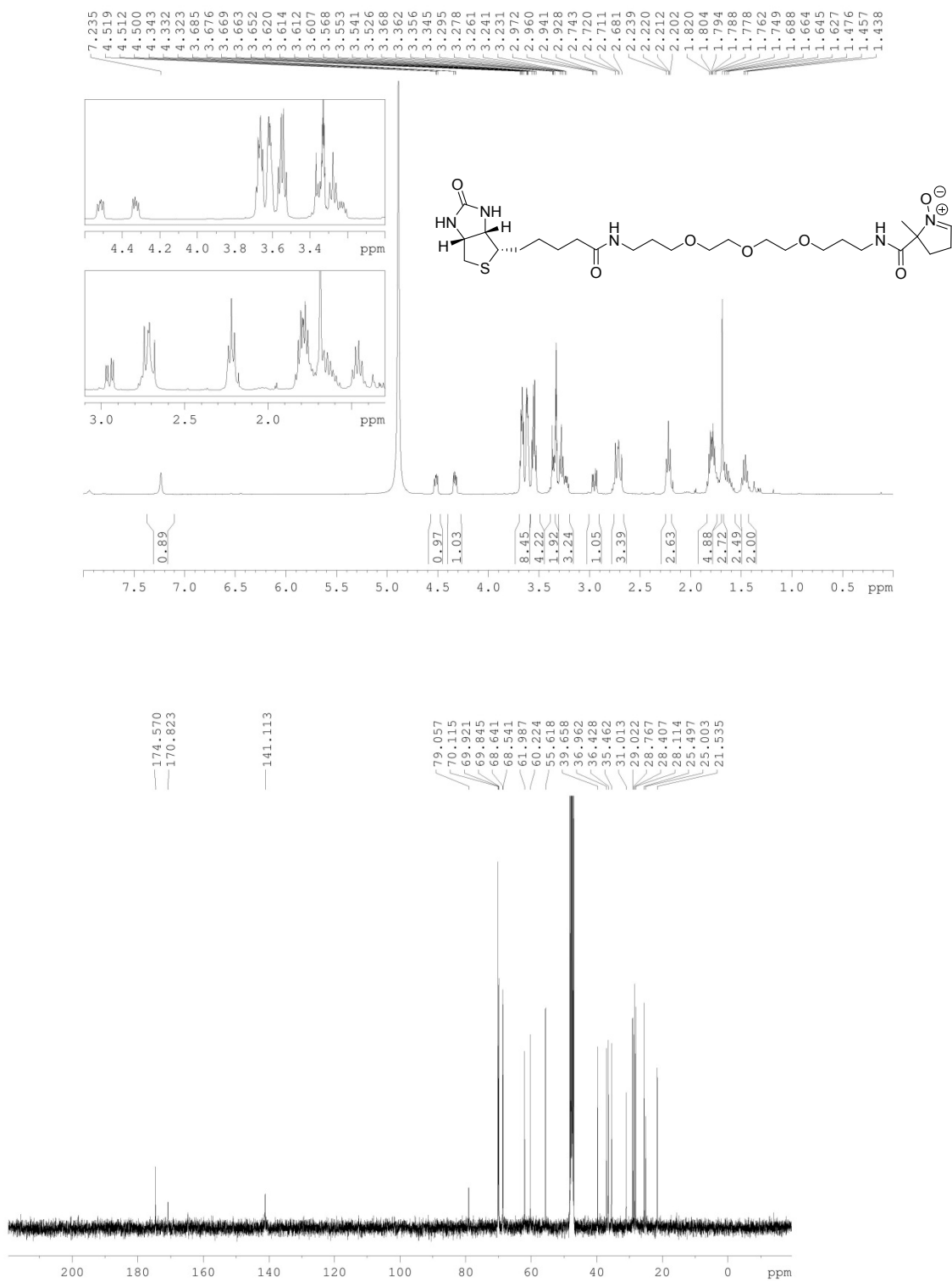
^1H and ^{13}C NMR Spectra of KDO-HMMPO



¹H and ¹³C NMR Spectra of KDO-alkyne



¹H and ¹³C NMR Spectra of Biotin-CMPO



References

- 1 D. C. Kennedy, C. S. McKay, M. C. B. Legault, D. C. Danielson, J. A. Blake, A. F. Pegoraro, A. Stolow, Z. Mester and J. P. Pezacki, *J. Am. Chem. Soc.*, 2011, **133**, 17993.
- 2 K. Stolze, N. Rohr-Udilova, A. Patel and T. Rosenau, *Bioorg. Med. Chem.*, 2011, **19**, 985.
- 3 (a) A. Dumont, A. Malleron, M. Awwad, S. Dukan and B. Vauzeilles, *Angew. Chem. Int. Ed.*, 2012, **51**, 3143; (b) R. Winzar, J. Philips and M. J. Kiefel, *Synlett*, 2010, **2010**, 583.
- 4 M. Slater, M. Snauko, F. Svec and J. M. J. Fréchet, *Anal. Chem.*, 2006, **78**, 4969.
- 5 (a) P. Tsai, K. Ichikawa, C. Mailer, S. Pou, H. J. Halpern, B. H. Robinson, R. Nielsen and G. M. Rosen, *J. Org. Chem.*, 2003, **68**, 7811; (b) S.-U. Kim, Y. Liu, K. M. Nash, J. L. Zweier, A. Rockenbauer and F. A. Villamena, *J. Am. Chem. Soc.*, 2010, **132**, 17157.
- 6 P.-T. Doulias, J. L. Greene, T. M. Greco, M. Tenopoulou, S. H. Seeholzer, R. L. Dunbrack and H. Ischiropoulos, *Proc Natl Acad Sci U S A*, 2010, **107**, 16958.
- 7 C. M. Tsai and C. E. Frasch, *Analytical biochemistry*, 1982, **119**, 115.