General information (protein analysis): The reverse phase HPLC traces, size-exclusion HPLC trace, SDS-PAGE gel, oxygen dissociation curves and mass spectrometry data presented in the Supporting Information section were acquired as previously described in the Experimental Section.

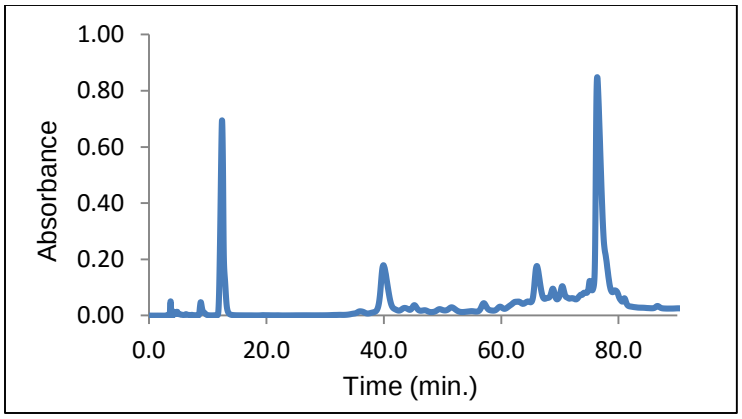


Figure 11. Reverse phase HPLC trace under dissociating conditions of the product of the reaction of α99-fumaryl-α99, β_2 with 5.5 eq. of cross-linker **1**. Peaks are due to elution of the heme (12 min.), β-subunits (40 min.), β cross-linked subunits (66 min.) and α cross-linked subunits (76 min.).

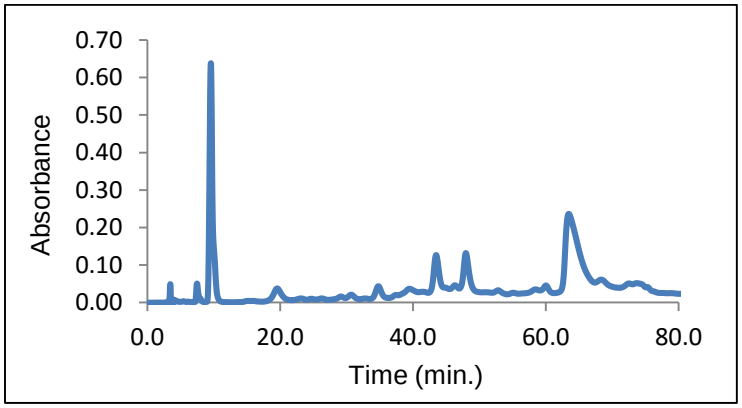


Figure 12. Reverse phase HPLC trace under dissociating conditions of the product of the reaction of α99-fumaryl-α99, β_2 with 16 eq. of cross-linker **1**. Peaks are due to elution of the heme (9 min.), β-subunits (18 min.), β cross-linked subunits (43 min.), β cross-linked subunits (48 min.) and α cross-linked subunits (63 min.).

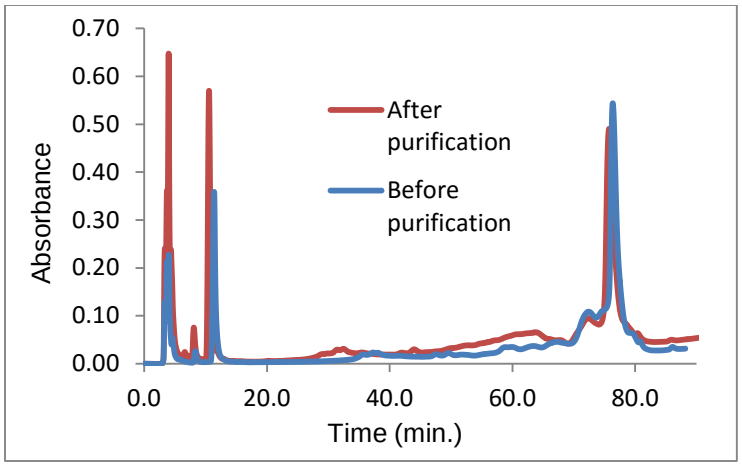


Figure 13. Reverse phase HPLC traces of the bis-tetramer containing mixture (the mixture presented in Figure 5) before and after purification under dissociating conditions. The heme elutes at 10 min. and the α cross-linked subunits elute at 76 min. The peak at 71 min. must be due to two species before purification: β82-azido-β82 (unreacted azide) and four covalently linked β-subunits (click product); and one species after purification: four covalently linked β-subunits (click product). It is typical for the tetra-β peak to appear broad.

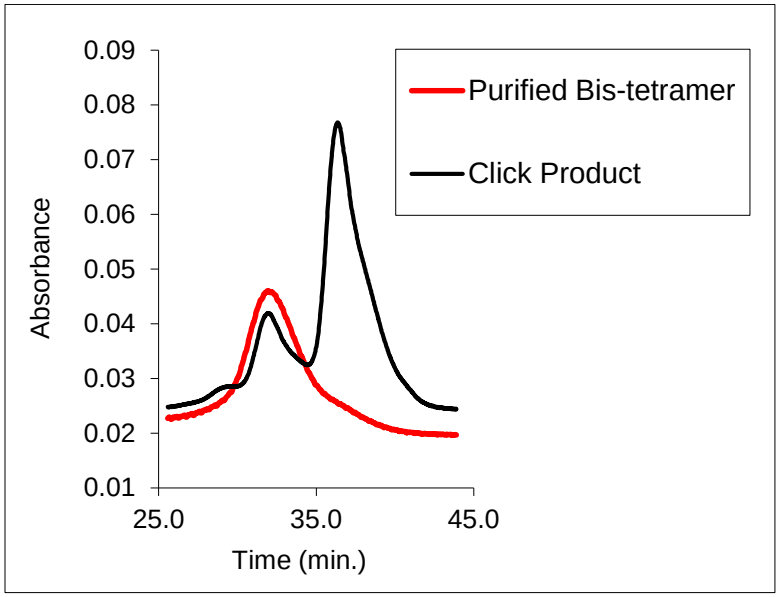


Figure 14. Size-exclusion HPLC traces (Tris-HCl buffer (37.5 mM, pH 7.4) containing 0.5 M magnesium chloride) of the click product mixture (as seen in Figure 5) and the purified bis-tetramer.

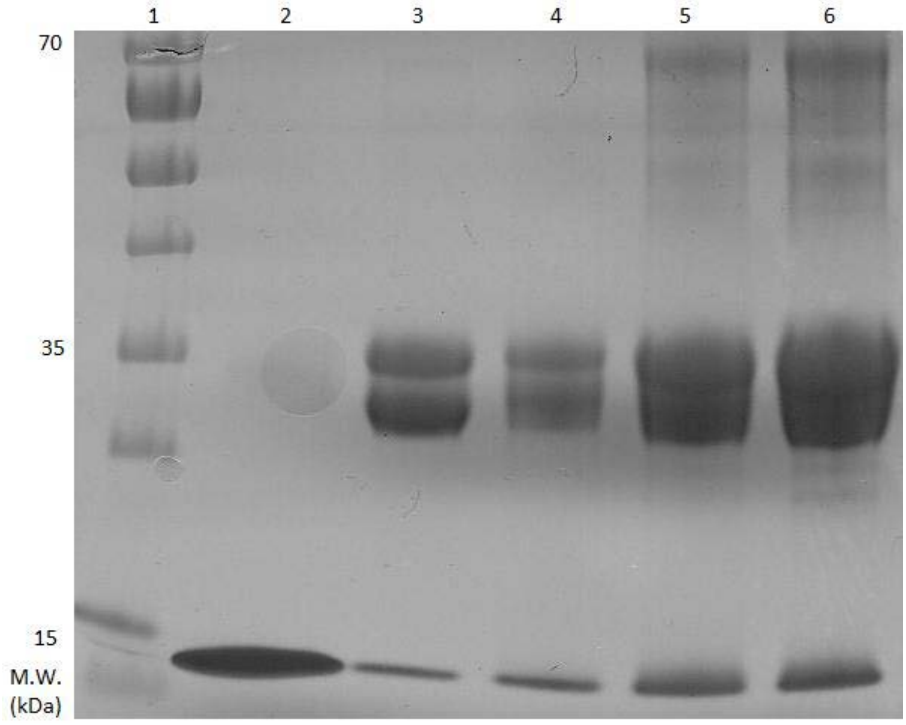


Figure 15. SDS-PAGE gel of protein ladder (lane 1); native Hb (lane 2); α99-fumaryl-α99, β82-trimesyl-β82 as in Figure 1 (lane 3); α99-fumaryl-α99, β82-azido-β82 containing mixture as in Figure 4 (lane 4); bis-tetramer containing mixture before purification (lanes 5 & 6).

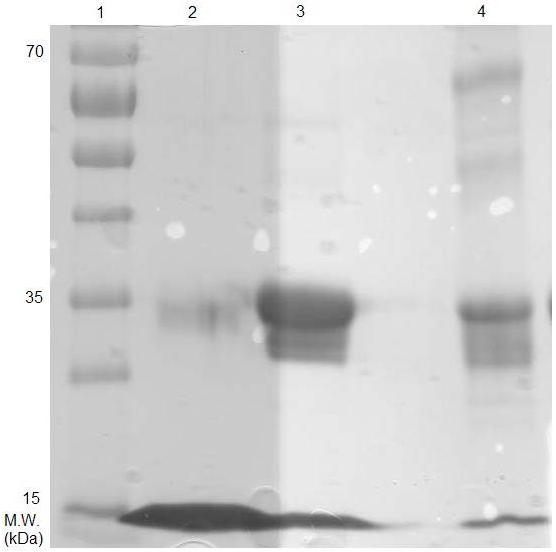


Figure 16. SDS-PAGE gel of protein ladder (lane 1); native Hb (lane 2); α99-fumaryl-α99, β_2 (lane 3); bis-tetramer ~90% pure by G200 analysis (lane 4).

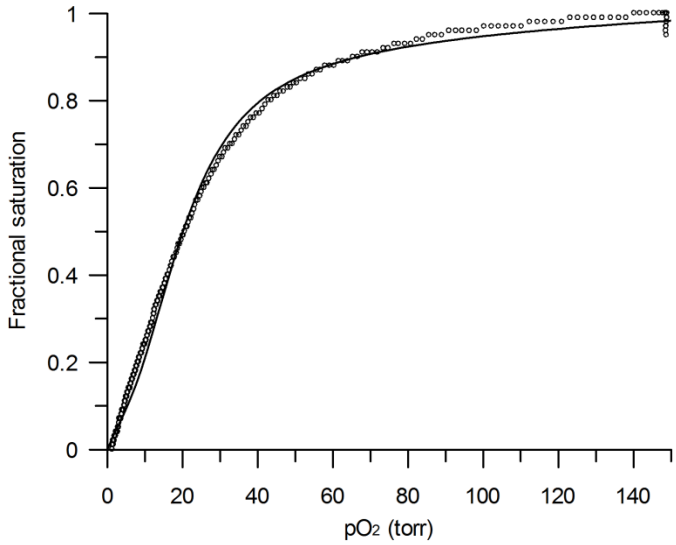


Figure 17. Oxygen dissociation curve of α99-fumaryl-α99, β82-trimesyl-β82 with the raw data (circles) superimposed with the fitted data (line).

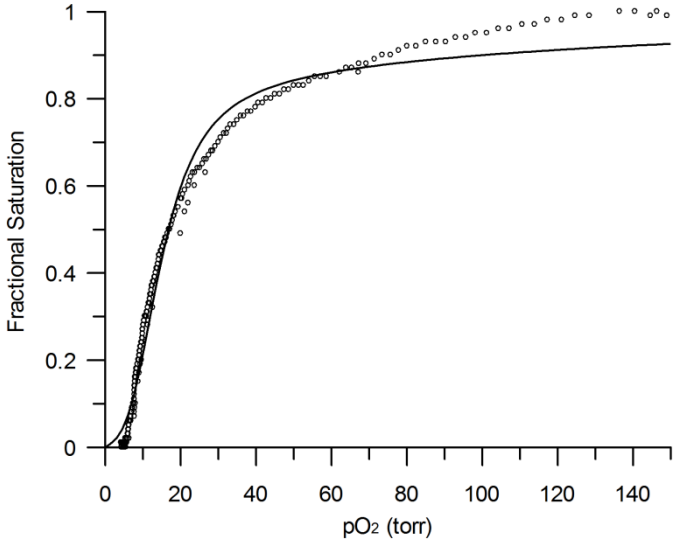


Figure 18. Oxygen dissociation curve of the bis-tetramer with the raw data (circles) superimposed with the fitted data (line). The fit may be improved by accounting for heterogeneous oxygen dissociation.

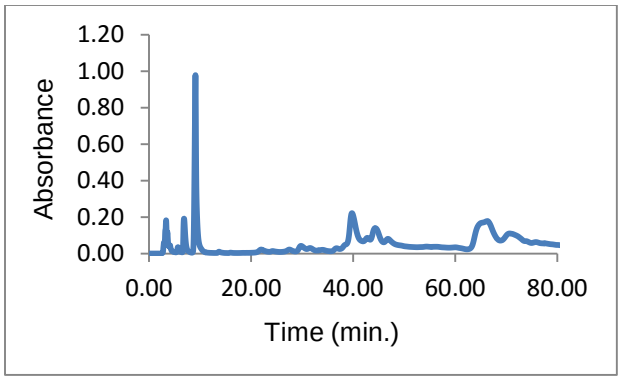


Figure 19. Reverse phase HPLC trace under dissociating conditions of the product mixture following nucleophilic substitution on non-hydrolyzed α99-fumaryl-α99, β82-trimesyl-β82 modified COHb. Peaks are due to elution of the heme (9 min.), β82-trimesyl-β82 (39 min.), β82-azido'-β82 (41 min.) and α cross-linked subunits (64 min.). See reference Yang, Y.; Kluger, R. Chem. Commun. 2010. 46, 7557-7559 for the reaction conditions.

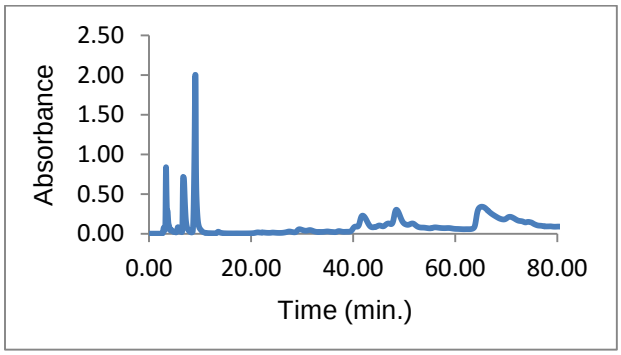


Figure 20. Reverse phase HPLC trace under dissociating conditions of the product mixture following nucleophilic substitution on non-hydrolyzed α99-fumaryl-α99, β82-trimesyl-β82 modified deoxyHb. Peaks are due to elution of the heme (9 min.), β82-trimesyl-β82 (41 min.), β82-azido'-β82 (48 min.) and α cross-linked subunits (64 min.). See reference Yang, Y.; Kluger, R. Chem. Commun. 2010. 46, 7557-7559 for the reaction conditions.

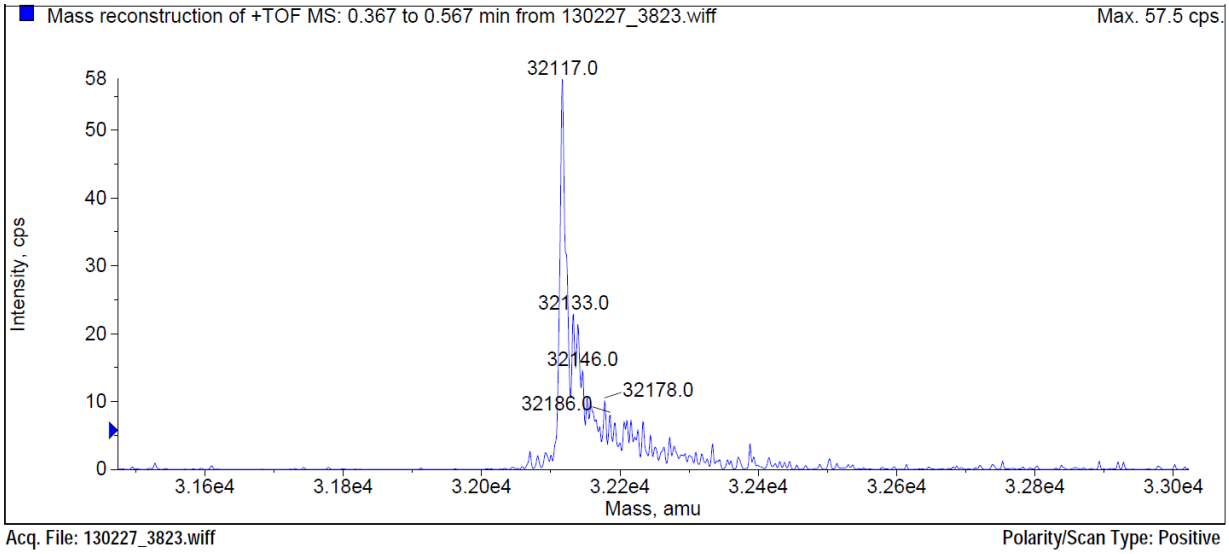


Figure 21. Mass spectrum of peak collected from reverse phase chromatography corresponding to β82-azido-β82 (see Figure 4, peak at 75 min.). (15867.22 Da – 1.01) x 2 β-subunits + $C_{16}H_9O_3N_4Br$ (385.19 Da) = 32117.61 Da.

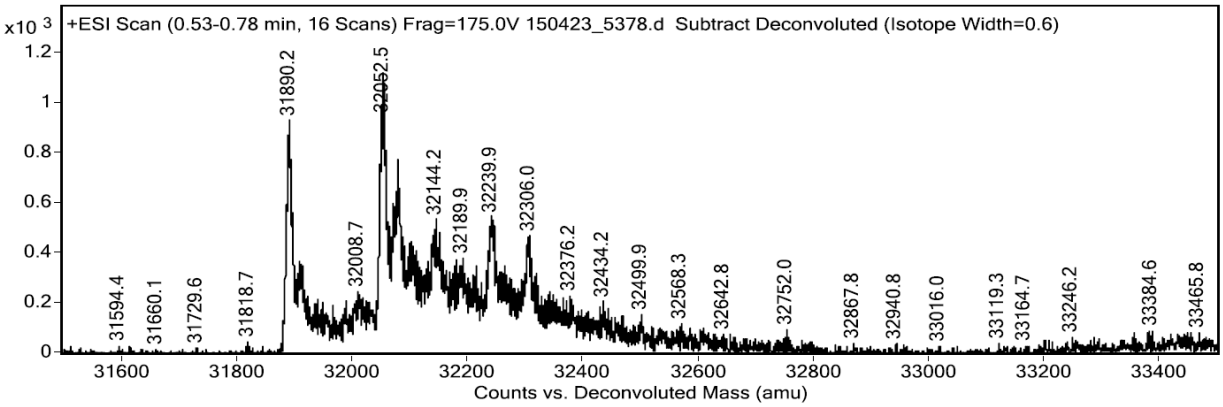
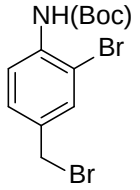


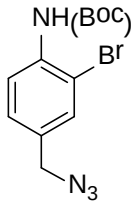
Figure 22. Mass spectrum of the peak collected from reverse phase HPLC as evidence of the β82-azido'-β82 modification (see Figure 21, peak at 41 min.). (15867.22 Da – 1.01) x 2 β-subunits + $C_{17}H_{12}O_3N_4$ (320.33 Da) = 32052.75 Da.

General methods (synthesis).
^{1}H NMR (400 and 500 MHz) and ^{13}C (100 and 125 MHz) spectra were recorded using a Varian Mercury spectrometer in $CDCl_3$ (internal standard, for ^{1}H residual $CHCl_3$ δ 7.24; for ^{13}C $CDCl_3$ δ 77.0) or CD_3OD (internal standard, for ^{1}H residual CD_2HOD δ 3.30; for ^{13}C CD_3OD δ 49.0). NMR data are reported using standard abbreviations: singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m) and broadened (b). Unless stated otherwise, all NMR spectra were recorded at 300 K. ESI mass spectra were acquired by the AIMS Lab (Department of Chemistry, University of Toronto). Compounds were purified by flash chromatography with Silica Gel 60 Å (230-400 μm mesh).

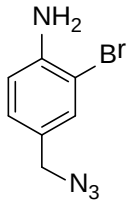
Synthesis of the azide functionalized cross-linker **2**.



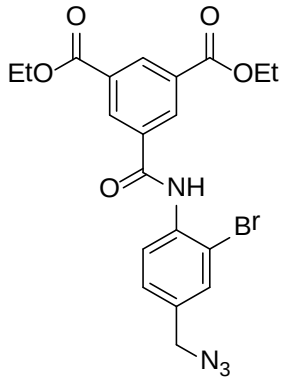
***tert*-Butyl (4-(bromomethyl)-2-bromophenyl)carbamate (3).**
tert-Butyl (2-bromo-4-methylphenyl)carbamate (1.43 g, 5 mmol) was dissolved in CCl_4 (10 mL). N-bromosuccinimide (0.98 g, 5.5 mmol) and benzoyl peroxide (24 mg, 0.1 mmol) were added. The mixture was heated to reflux and stirred for 4 h. The organic phase was washed with saturated aq. $NaHCO_3$, dried with $MgSO_{4(s)}$, filtered then concentrated to give an oil. Purification by column chromatography (1:1 CH_2Cl_2:hexanes) gave the pale yellow product **3** (86%). ^{1}H NMR ($CDCl_3$, 400 MHz): 8.13 (1H, d, J = 8.5 Hz, Ar), 7.53 (1H, d, J = 2.2 Hz, Ar), 7.29 (1H, dd, J = 8.5, 2.2 Hz, Ar), 7.03 (1H, br-s, NH), 4.40 (2H, s, CH_2-Br), 1.53 (9H, s, ^{t}Bu). ^{13}C NMR ($CDCl_3$, 100 MHz): 152.20 (NHCOO^{t}Bu or Boc), 136.45 (Ar), 133.27 (Ar), 132.79 (Ar), 129.12 (Ar), 119.90 (Ar), 112.08 (Ar), 81.39 (^{t}Bu), 32.25 (CH_2-Br), 28.33 (^{t}Bu). ESIMS calcd for $C_{12}H_{15}NBr_2$ $[M+H]^+$: 331.0, found 331.0.



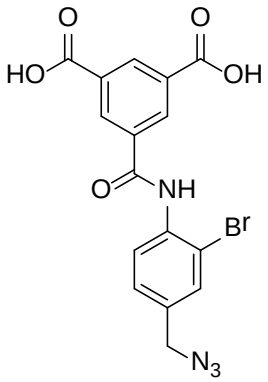
***tert*-Butyl (4-(azidomethyl)-2-bromophenyl)carbamate (4).**
The oil **3** (500 mg, 1.7 mmol) was dissolved in anhydrous DMF (5 mL). NaN_3 (6 eq., 650 mg, 10 mmol) was added and this mixture was stirred overnight at 65 °C. Diethyl ether was added (50 mL) and the organic phase was washed with H_2O (50 mL), dried over $MgSO_{4(s)}$, filtered then concentrated to give a yellow oil. Purification by column chromatography (25% EtOAc:hexanes) gave the product **4** (94%). ^{1}H NMR ($CDCl_3$, 400 MHz): 8.17 (1H, d, J = 8.5 Hz, Ar), 7.46 (1H, d, J = 2.0 Hz, Ar), 7.22 (1H, dd, J = 8.5, 2.0 Hz, Ar), 7.03 (1H, br-s, NH), 4.24 (2H, s, CH_2-N_3), 1.53 (9H, s, ^{t}Bu). ^{13}C NMR ($CDCl_3$, 100 MHz): 152.20 (NHCOO^{t}Bu or Boc), 136.31 (Ar), 131.86 (Ar), 128.15 (Ar), 120.01 (Ar), 112.35 (Ar), 112.23 (Ar), 81.23 (^{t}Bu), 53.60 (CH_2-N_3), 28.21 (^{t}Bu). ESIMS calcd for $C_{12}H_{15}N_4Br$ $[M+H]^+$: 294.1, found 294.1.



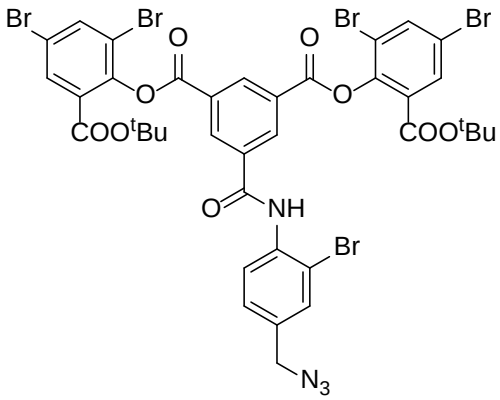
4-(Azidomethyl)-2-bromoaniline (5). Compound **4** (500 mg, 1.53 mmol) was dissolved in anhydrous CH_2Cl_2 (5 mL, dried over $MgSO_{4(s)}$) and this solution was cooled to 0 °C. TFA (1 mL) was added. This mixture was stirred for one hour then saturated aq. $NaHCO_3$ was added until a pH strip indicated pH 8. The organic phase was extracted, washed with $NaCl_{(aq)}$, dried over $MgSO_{4(s)}$, filtered and concentrated to give a yellow oil. Purification by column chromatography (25% EtOAc:hexanes) gave product **5** (70%). ^{1}H NMR ($CDCl_3$, 500 MHz): 7.38 (1H, d, J = 2.1 Hz, Ar), 7.06 (1H, dd, J = 8.2, 2.1 Hz, Ar), 6.76 (1H, d, J = 8.2 Hz, Ar), 4.19 (2H, s, CH_2-N_3). ^{13}C NMR ($CDCl_3$, 125 MHz): 144.33 (Ar), 132.79 (Ar), 128.78 (Ar), 126.36 (Ar), 115.83 (Ar), 109.24 (Ar), 54.10 (CH_2-N_3). ESIMS calcd for $C_7H_7N_4Br$ $[M+H]^+$: 226.0, found 226.0.



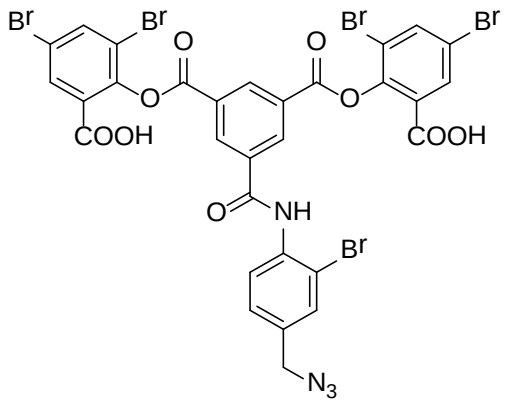
Diethyl 5-((4-(azidomethyl)-2-bromophenyl)carbamoyl)isophthalate (6). Diethyl 1,3,5-benzenetricarboxylate (772 mg, 2.9 mmol) was refluxed in $SOCl_2$ (4 mL) for 4 h. This mixture was concentrated to give an oil residue, which was subsequently dissolved in anhydrous THF (5 mL, dried over $MgSO_{4(s)}$). This solution was cooled to 0 °C then added to a solution of amine **5** (427 mg, 1.9 mmol) and DMAP (257 mg, 2.1 mmol) in THF (10 mL, dried over $MgSO_{4(s)}$) also at 0 °C. The mixture was stirred overnight at room temperature then filtered under vacuum and washed with EtOAc. The organic phase was washed with H_2O and $NaCl_{(aq)}$ then dried over $MgSO_{4(s)}$, filtered and concentrated to give an oil. Purification by column chromatography (25% EtOAc:hexanes) gave the construct **6** (65%). ^{1}H NMR ($CDCl_3$, 500 MHz): 8.88 (1H, t, J = 1.6 Hz, Ar), 8.77 (2H, d, J = 1.6 Hz, Ar), 8.53 (2H, m, Ar+ NH), 7.59 (1H, d, J = 2.1 Hz, Ar), 7.35 (1H, dd, J = 8.5, 2.1 Hz, Ar), 4.46 (4H, q, J = 7.1 Hz, CH_2-O), 4.35 (2H, s, CH_2-N_3), 1.45 (6H, t, J = 7.1 Hz, CH_3CH_2-O). ^{13}C NMR ($CDCl_3$, 125 MHz): 165.02 (CO_2Et), 163.66 (CONH), 135.44 (Ar), 135.30 (Ar), 133.98 (Ar), 133.34 (Ar), 132.14 (Ar), 132.13 (Ar), 132.03 (Ar), 128.52 (Ar), 122.24 (Ar), 114.38 (Ar), 62.04 (CH_2-O), 53.80 (CH_2-N_3), 14.46 (CH_3CH_2-O). ESIMS calcd for $C_{20}H_{19}BrN_4O_5$ $[M+H]^+$: 474.1, found 474.1.



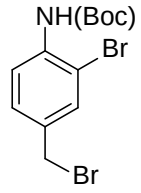
5-((4-(azidomethyl)-2-bromophenyl)carbamoyl)isophthalic acid (7).
The protected ester **6** (237 mg, 0.50 mmol) was dissolved in MeOH/THF (6 mL, 1:1 by volume). A solution of KOH in H_2O (0.5 g/mL, 2 mL) was added. The mixture was stirred at room temperature for 1 h. Aq. HCl (2.0 M) was added until the pH of the solution reached ~2-3. EtOAc (10 mL) was added and the organic layer was washed with H_2O, dried over $MgSO_{4(s)}$ and concentrated to give the deprotected diacid **7** as a white solid (89%). ^{1}H NMR (CD_3OD, 500 MHz): 8.83 (4H, m, Ar+ NH), 7.74 (2H, m, Ar), 7.43 (1H, dd, J = 7.4, 2.1 Hz, Ar), 4.43 (2H, s, CH_2-N_3). ^{13}C NMR (CD_3OD, 125 MHz): 167.90 (CO_2H), 167.12 (CONH), 137.69 (Ar), 136.99 (Ar), 136.43 (Ar), 134.82 (Ar), 133.79 (Ar), 133.70 (Ar), 133.34 (Ar), 129.19 (Ar), 129.08 (Ar), 121.24 (Ar), 54.34 (CH_2-N_3). ESIMS calcd for $C_{16}H_{11}BrN_4O_5$ $[M-H]^-$: 418.0, found 417.0.



1,3-bis(2,4-Dibromo-6-(tert-butoxycarbonyl)phenyl)-5-((4-(azidomethyl)-2-bromophenyl)carbamoyl)isophthalate (8). The diacid **7** (84 mg, 0.20 mmol), *tert*-butyl 3,5-dibromosalicylic acid (142 mg, 0.40 mmol) and DMAP (4.9 mg, 40 μmol) were dissolved in anhydrous THF (20 mL, dried over $MgSO_{4(s)}$). The solution was cooled to 0 °C in an ice/water bath. EDC (96 mg, 0.50 mmol) in DCM (2 mL) was added. The mixture was stirred at 0 °C for 10 min then overnight at room temperature. EtOAc was added and the organic layer was washed with saturated aq. $NaHCO_3$, dried over $MgSO_{4(s)}$ then concentrated. Purification by column chromatography (25% EtOAc:hexanes) gave the protected diester **8** (70%). ^{1}H NMR ($CDCl_3$, 500 MHz): 9.24 (1H, t, J = 1.6 Hz, Ar), 9.05 (2H, d, J = 1.6 Hz, Ar), 8.60 (1H, br-s, NH), 8.55 (1H, d, J = 8.4 Hz, Ar), 8.05 (2H, d, J = 2.4 Hz, Ar), 7.96 (2H, d, J = 2.4 Hz, Ar), 7.60 (1H, d, J = 2.1 Hz, Ar), 7.36 (1H, dd, J = 8.4, 2.1 Hz, Ar), 4.35 (2H, s, CH_2-N_3), 1.43 (18H, s, ^{t}Bu). ^{13}C NMR ($CDCl_3$, 125 MHz): 163.03 (CONH), 162.02 (CO_2R), 161.75 ($CO_2{}^t$Bu), 146.64 (Ar), 139.08 (Ar), 136.20 (Ar), 135.43 (Ar), 135.30 (Ar), 133.88 (Ar), 133.84 (Ar), 133.53 (Ar), 132.05 (Ar), 130.98 (Ar), 128.73 (Ar), 128.56 (Ar), 122.24 (Ar), 119.95 (Ar), 119.30 (Ar), 114.41 (Ar), 83.41 ($CO_2{}^t$Bu), 53.79 (CH_2-N_3), 28.15 (^{t}Bu). ESIMS calcd for $C_{38}H_{31}Br_5N_4O_9$ $[M+H]^+$: 1081.8, found 1081.7.



1,3-bis(2,4-Dibromo-6-(carboxyphenyl)-5-((4-(azidomethyl)-2-bromophenyl)carbamoyl)isophthalate (2). The protected ester **8** (100 mg, 93.8 μmol) was dissolved in CH_2Cl_2 (4 mL) and TFA (1 mL) was added. The mixture was stirred at room temperature for one hour then concentrated under vacuum to give the activated cross-linker **2** (90%). ^{1}H NMR (CD_3OD, 500 MHz): 9.52 (3H, m, Ar), 8.20 (2H, d, J = 2.3 Hz, Ar), 8.17 (2H, d, J = 2.3 Hz, Ar), 7.73 (2H, m, Ar+ NH), 7.43 (1H, dd, J = 8.5, 2.3 Hz, Ar), 4.43 (2H, s, CH_2-N_3). ^{13}C NMR (CD_3OD, 125 MHz): 166.17 (CONH), 165.00 (CO_2R), 163.65 (CO_2H), 148.70 (Ar), 140.53 (Ar), 137.89 (Ar), 137.25 (Ar), 136.84 (Ar), 135.62 (Ar), 135.32 (Ar), 135.13 (Ar), 133.71 (Ar), 131.86 (Ar), 129.35 (Ar), 129.08 (Ar), 128.60 (Ar), 121.37 (Ar), 120.73 (Ar), 120.57 (Ar), 54.32 (CH_2-N_3). ESIMS calcd for $C_{30}H_{15}Br_5N_4O_9$ $[M-H]^-$: 969.7, found 969.8.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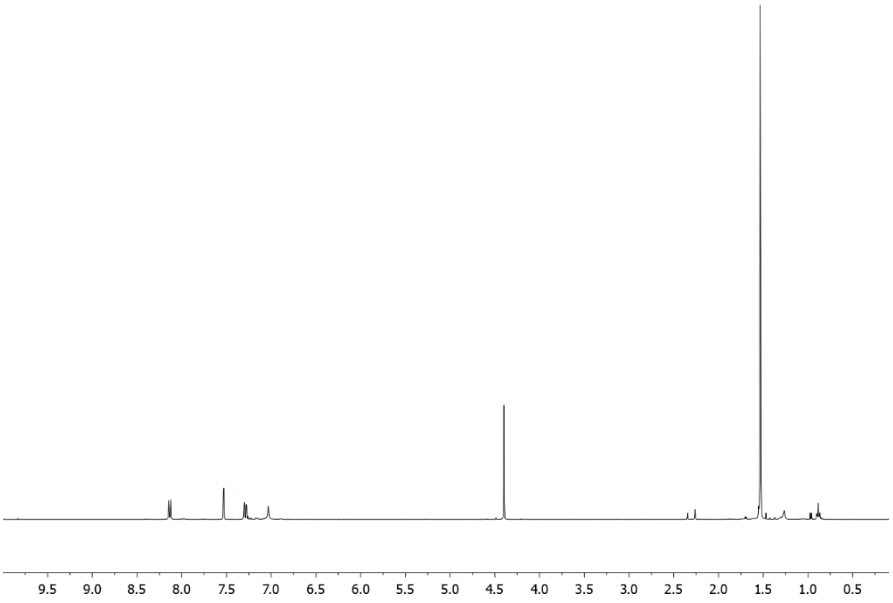
Br
Br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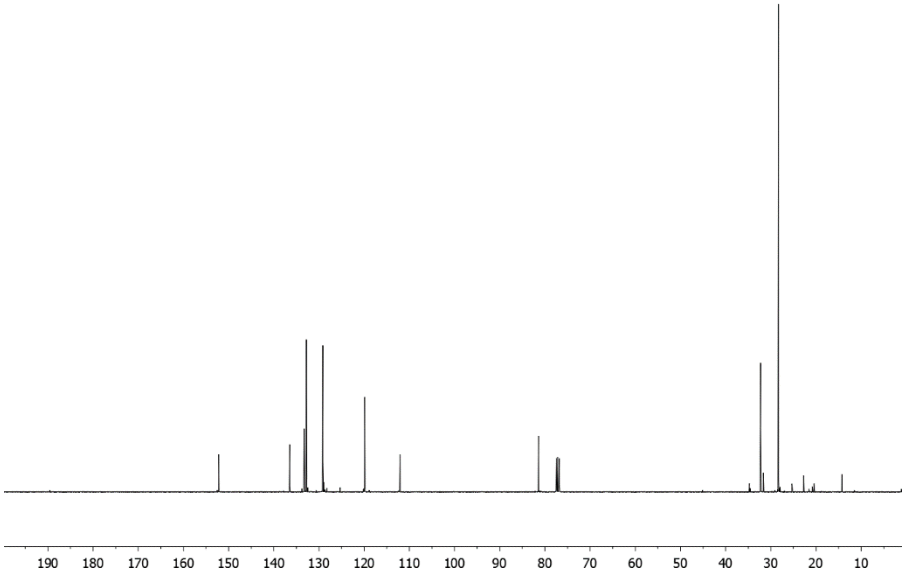
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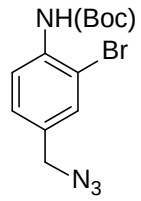
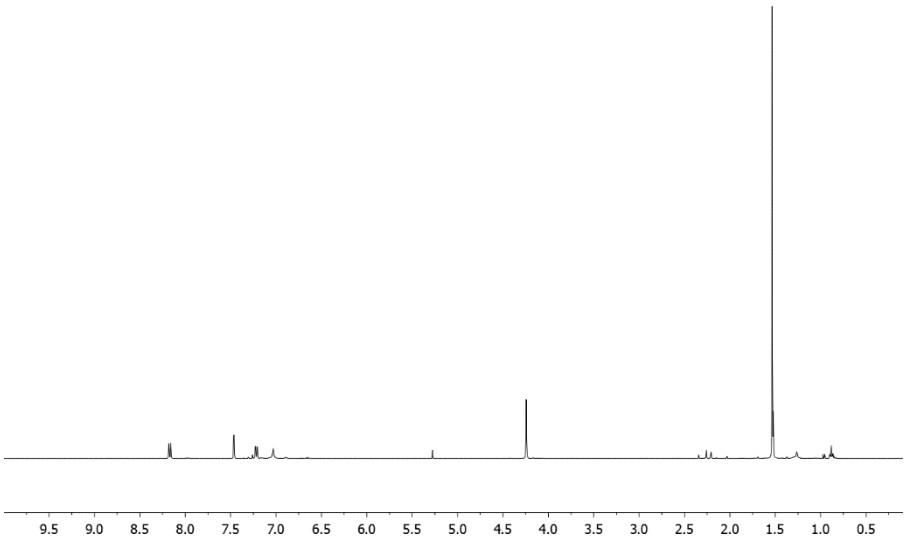

NH(Boc)
Br
N3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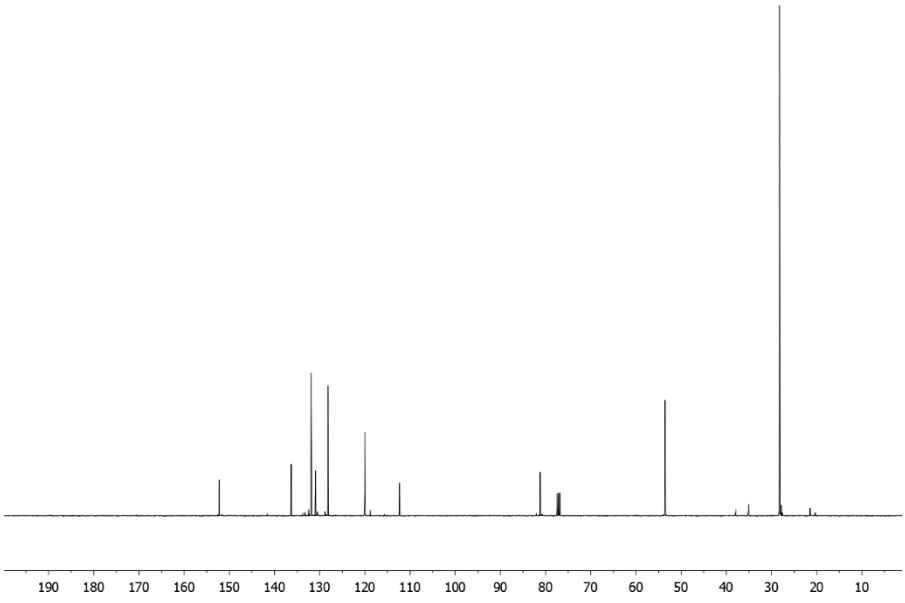
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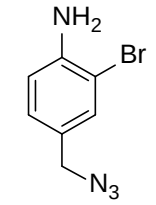
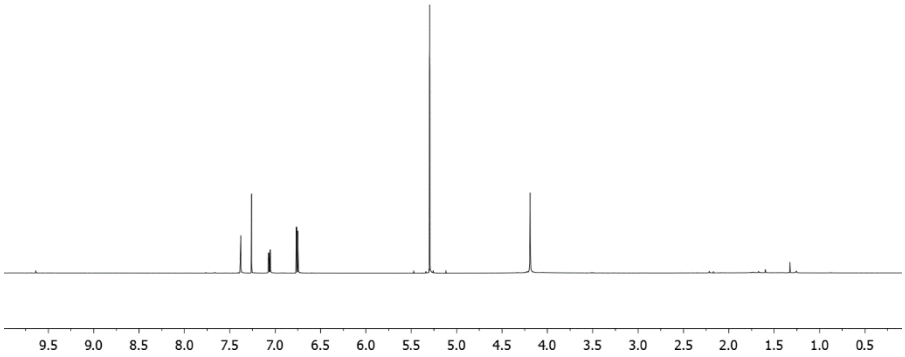

NH2
Br
N3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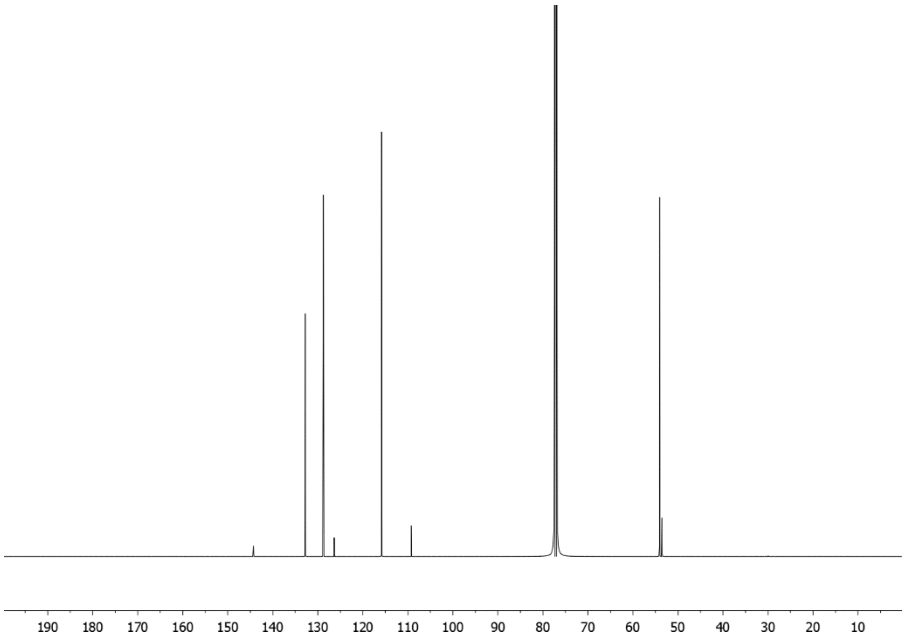
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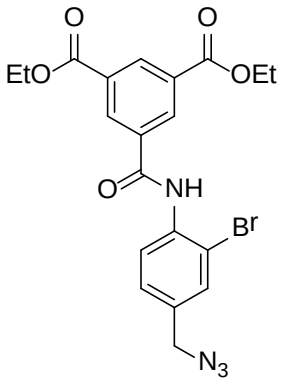
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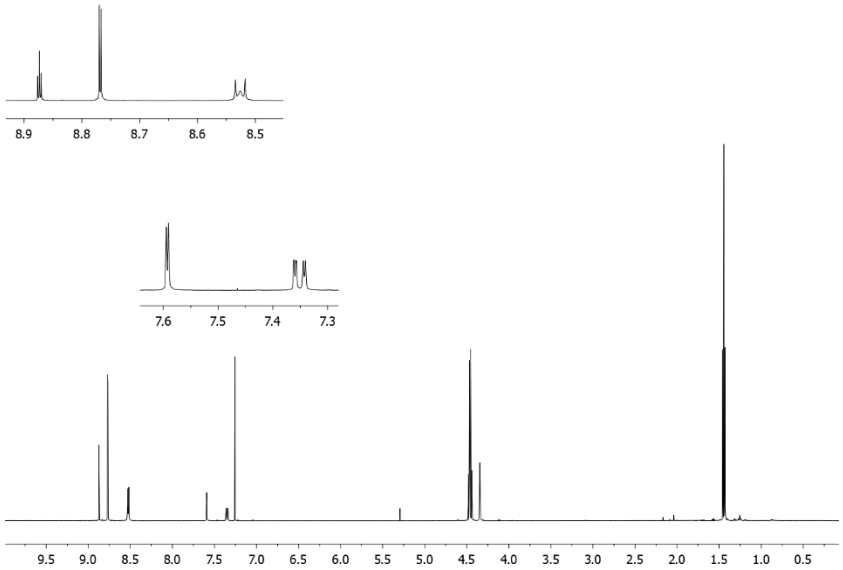
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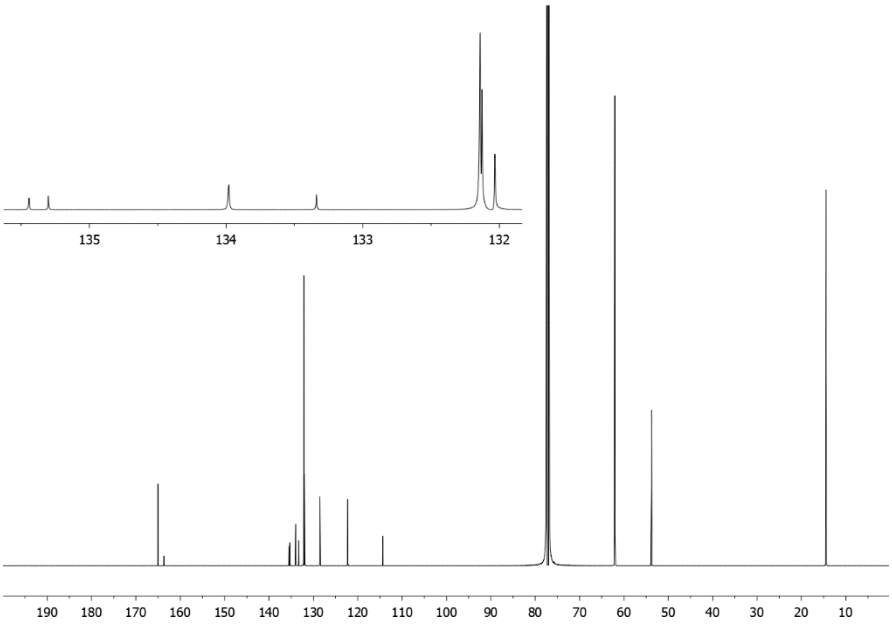
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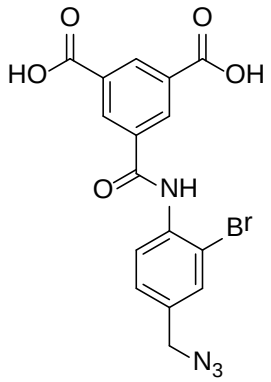
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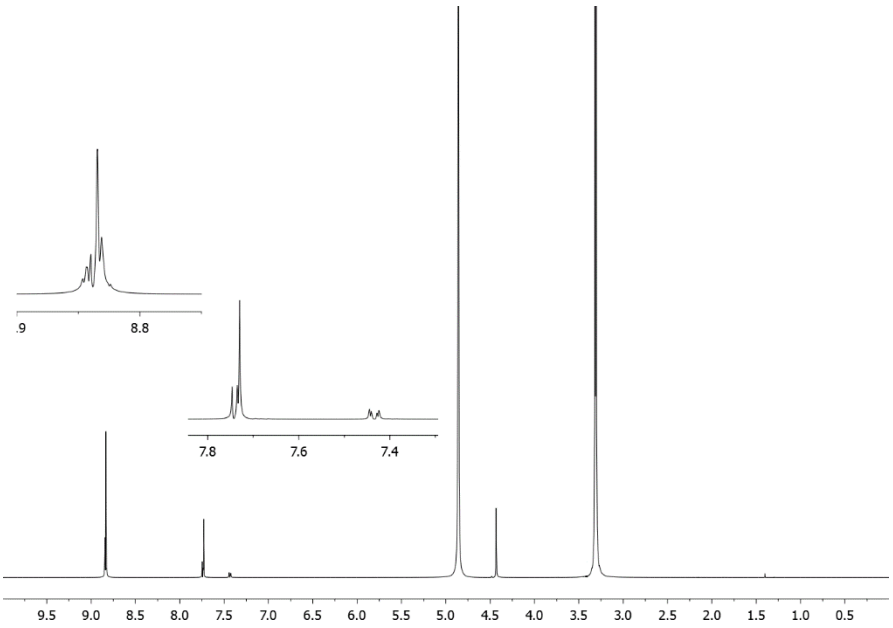
HO
OH
O
NH
Br
N3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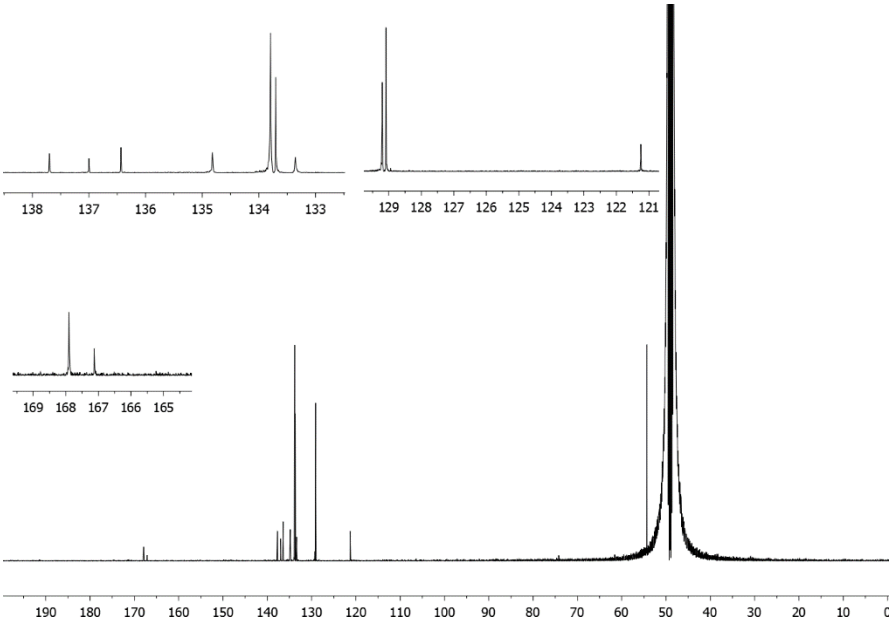
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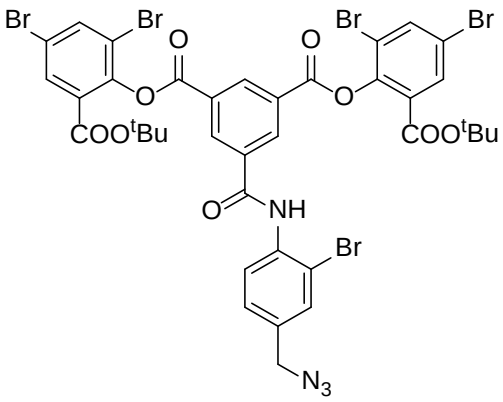
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O
COO^{t}Bu
COO^{t}Bu
O
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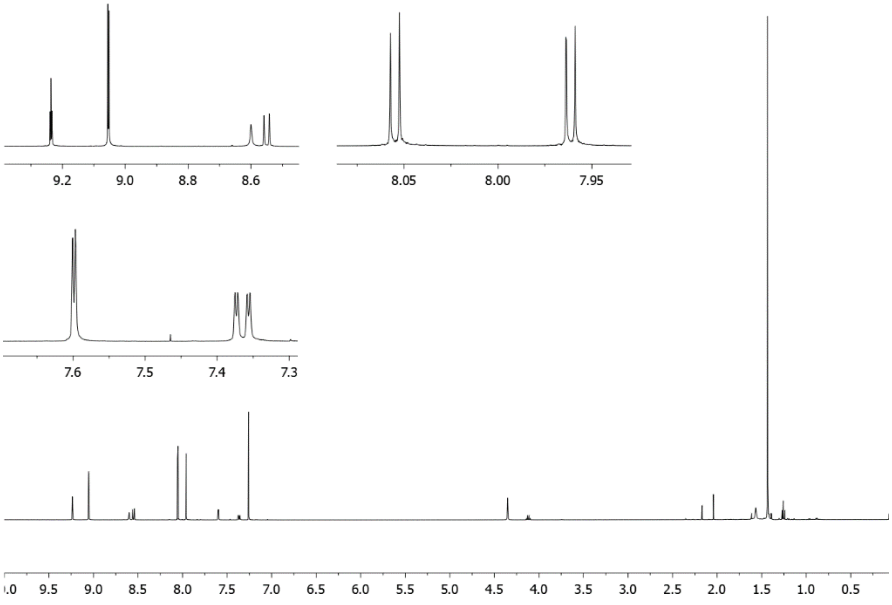
Br
N_3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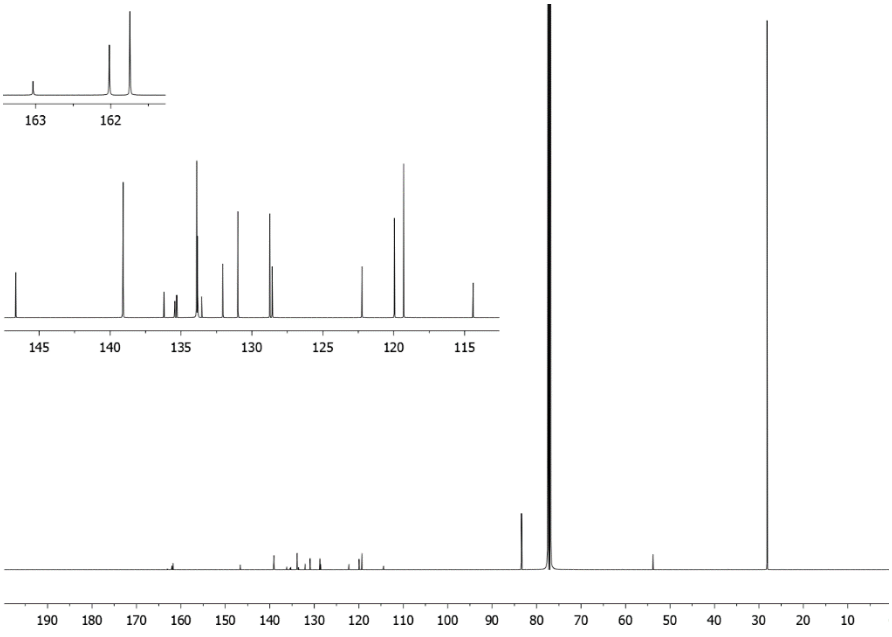
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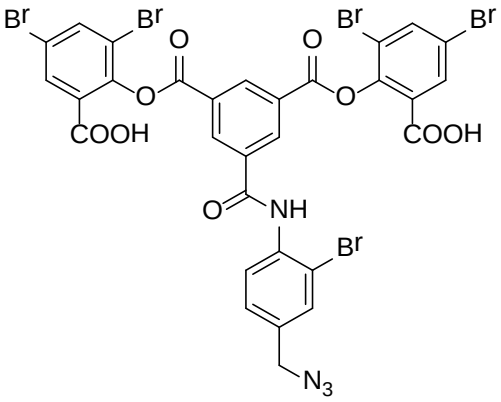
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COOH
COOH
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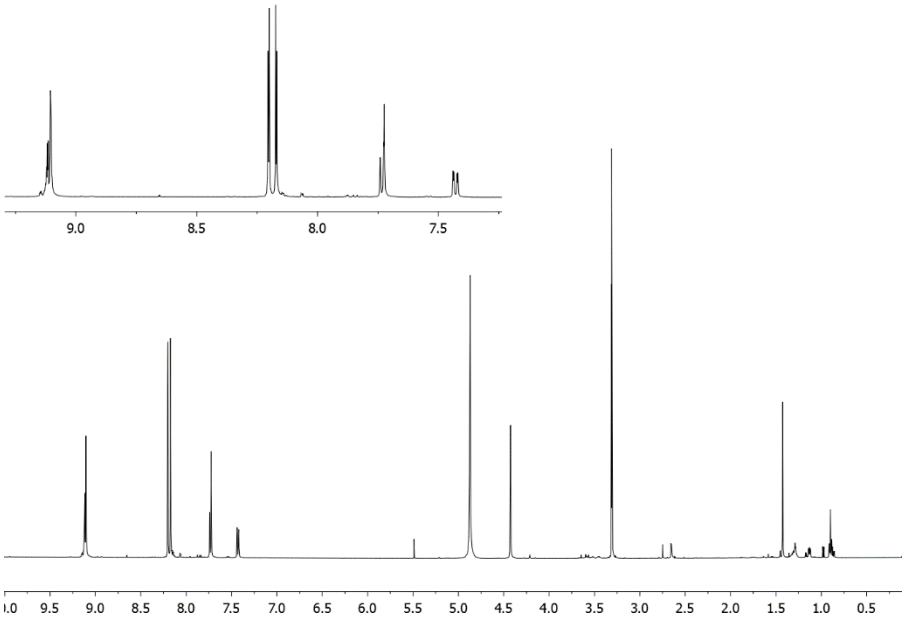
NH
Br
N3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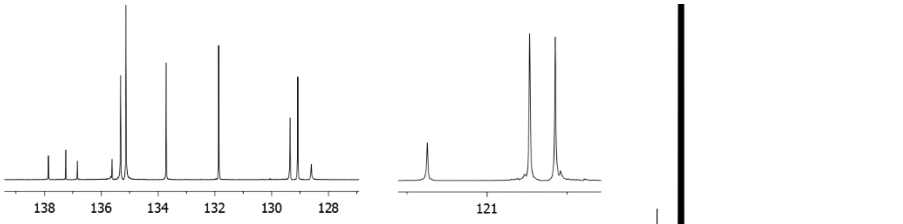
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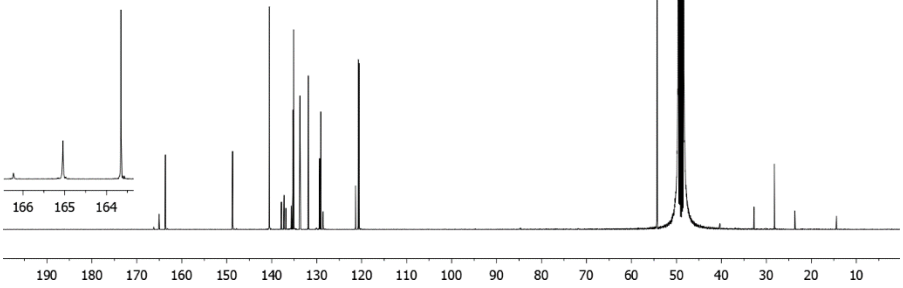
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