Chemical Control Over Immune Recognition: A Class of Antibody-Recruiting Molecules (ARMs) that Target Prostate Cancer

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Supporting Information

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General Information

Synthesis: Unless otherwise stated, all reactions were carried out in flame-dried glassware under a nitrogen atmosphere. All reagents were purchased from commercial suppliers and used without further purification except the following: triethylamine was distilled over calcium hydride; CH_2Cl_2, PhMe, DMF, and THF were purified using a solvent dispensing system;[1] Water was purified using a Milli-Q purification system. Infrared (**IR**) spectra bands are characterized as broad (br), strong (s), medium (m) and weak (w). **^{1}H NMR** chemical shifts are reported with the solvent residual peak as the internal standard ($CDCl_3$ δ 7.26 ppm or CD_3OD δ 3.31 ppm). Data are reported as follows: chemical shift, integration, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broad, m = multiplet), and coupling constants (Hz). **^{13}C NMR** chemical shifts are reported in ppm with the solvent as an internal reference ($CDCl_3$ δ 77.2 ppm or CD_3OD δ 49.00 ppm or d-DMSO δ 39.5 ppm). Azido polyethylene glycol derivatives $N_3(C_2H_2O)_nH$ were synthesized using standard chemistry[2] from the corresponding polyethylene glycol of the highest oligomer purity commercially available through Aldrich (all cases $\geq$ 90% major oligomer).

Biology: LNCaP cells (ATCC, # CRL-1740) were cultured in RPMI Medium 1640 + 10% FBS + 100 U/mL penicillin/streptomycin at 37°C, 5% CO_2. DU145 cells (ATCC, # HTB-81) were cultured in MEM + 10% FBS + 100 U/mL penicillin/streptomycin at 37°C, 5% CO_2.

Buffer Tris-A (**TBS-A**) consists of 25 mM Tris-HCl, 150 mM NaCl, 1.5% BSA, 5 mM Glucose, 1.5 mM $MgCl_2$, pH 7.2.

Buffer Tris-B (**TBS-B**) consists of 25 mM Tris-HCl and 150 mM NaCl, pH 7.2.

ADCC medium consists of phenol red free RPMI Medium 1640 + 5% FBS + 100 U/mL penicillin/streptomycin.

[1] Pangborn, A.B.; Giardello, M.A.; Grubbs, R.H.; Rosen, R.K.; Timmers, F.J. *Organometallics* **1996**, *15*, 1518.
[2] Zych, A.; Iverson, B. *J. Amer. Chem. Soc.* **2000**, *37*, 8898-8909.

Molecular Modeling

No structural data was available for urea based PSMA ligands at the outset of this project. Consequently a model of urea based ligand bound to PSMA consistent with experimental data was generated on the basis of the crystal structure of PSMA in complex with a phosphonate based ligand (PDB 3BI1).[3] The procedure involved placement of the urea core in the binding site of PSMA and energy minimization of the resulting complex with the de-nova design program BOMB (Biochemical and Organic Model Builder).[4] BOMB was then used to screen libraries of urea-analogs that would position substituents in the S2 pocket of PSMA. This exercise identified 1,4-substituted-1,2,3-triazoles as a promising design owing to 1) favorable energetic interactions with Arg463 and Tyr700 2) the orientation of the ring that project 4-substituents away from the binding site 3) the ease of synthesis.

Since PSMA functions as a homodimer in vivo, the dimer was next modeled from the complex of monomeric PSMA with the cell binding terminus (CBT) moiety of the ARM-Ps generated by BOMB. The x-ray structure of the Fv region of SPE-7, in complex with DNP-Serine (PDB 1OAU) was then manually docked near the PSMA dimer.[5] DNP-Serine was then replaced by the antibody binding terminus (ABT) of the ARM-P scaffold**.**

The program FIRST was then used to determine the flexibility of the modeled ternary complex by graph theory.[6] The FRODA module of FIRST was then used to perform constrained geometric simulations of the ternary complex.[7] Targeted dynamics options were used to instruct the program to bring the ABT and CBT ends of the ARM-P molecules as close as possible without incurring steric clashes between the two proteins. The FRODA simulations were performed in two stages. First the PSMA-CBT and SPE-7-ABT complexes were treated as rigid bodies and brought into close contact using 10,000 configurations. Next induced fit at the protein interfaces was performed using the default protein flexibility settings of FRODA (energy cutoff -1.0 kcal/mol for hydrogen bond detection and hydrophobic function H3) and generating 100,000 configurations. The simulations were repeated from different initial orientations of the Fab domain to assess convergence.

Analysis of the simulations suggested that the separation between the ABT and CBT fragments should be at minimum ~20 Å. Bringing the ABT and CBT fragments closer would create steric clashes that could only be resolved by breaking native contacts in both proteins. At this separation the Fv region of SPE-7 must approach PSMA sideways at approximately 45 degrees from the bilayer plane. Six oxyethylene units in extended conformation would be required to span this distance. However close contact between the two proteins would require dehydration of the protein-protein interface and this was judged energetically

[3] Barinka, C.; Byun, Y.; Dusich, C. L.; Banerjee, S. R.; Chen, Y.; Castanares, M.; Kozikowski, A. P.; Mease, R. C.; Pomper, M. G.; Lubkowski, J. *J. Med. Chem.* **2008**, *51*, 7737-7743.
[4] Jorgensen, W. L. *Acc. Chem. Res.* **2009**, *42*, 724-733.
[5] James, L. C.; Roversi, P.; Tawfik, D. S. *Science* **2003**, *299*, 1362-1367.
[6] Jacobs, D. J.; Rader, A. J.; Kuhn, L. A.; Thorpe, M. F. *Proteins: Struct., Funct., Genet.* **2001**, *44*, 150-165.
[7] Wells, S.; Menor, S.; Hespenheide, B.; Thorpe, M. F. *Phys. Biol.* **2005**, *24*, S127-S136.

unfavorable given that many solvent-exposed polar side chains would not be able to engage in hydrogen bonding interactions with the protein partner. It was therefore judged preferable to maintain the two proteins interfaces at ~5-10 Å separation so each would retain 1-2 hydration shell. The simulations revealed this would require a separation of ca. 25 Å between the ABT and CBT moieties that could be covered by 7 oxyethylene units in a completely extended conformation (Figure S1). Finally, it was judged preferable to overshoot this estimate to avoid overly rigidifying the linker, which would be expected to contribute negatively to the entropy of binding.

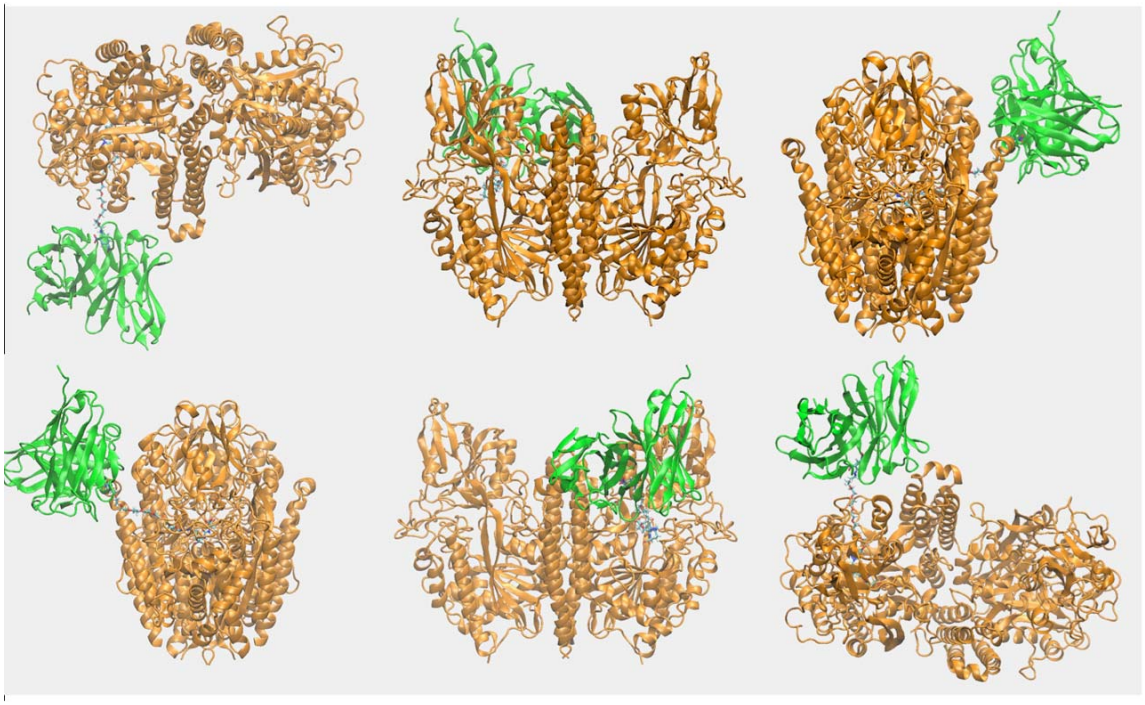
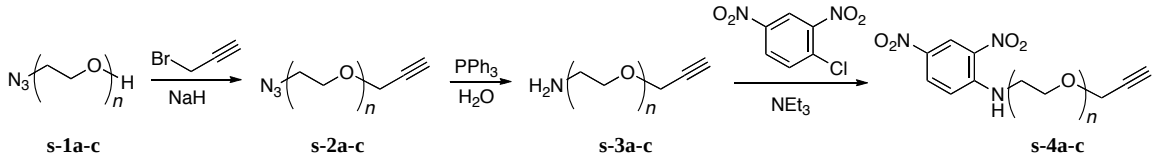


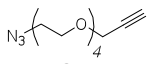
Figure S1. Representative snapshot of the modeled Anti-DNP Fv region, ARM-P8, PSMA ternary complex seen from above (top left panel), sideways and below (bottom right panel) the bilayer plane. The snapshot was taken from a FRODA simulation.

Synthesis



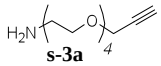
Scheme S1. General synthesis of DNP-PEG$_n$-yne from corresponding azido alcohol. **a**, n=4; **b**, n=6; **c**, n=8.

1-azido-3,6,9,12-tetraoxapentadec-14-yne (s-2a)


s-2a

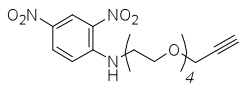
2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethanol (**s-1a**) (7.55 g, 34 mmol, 1.0 equiv.) was dissolved in DMF (150mL) and the resulting solution was cooled to 0 ^{o}C. Sodium hydride (979 mg, 40.8 mmol, 2.0 equiv.) was added slowly, followed by dropwise addition of propargyl bromide (80% in PhMe, 7.4 mL, 68 mmol, 1.2 equiv.). The reaction ran for 3 h at rt, at which time it was found complete by thin layer chromatography. The solution was then concentrated and chromatographed (3 cm x 25 cm Silica, 30% EtOAc in hexanes) to yield **1-azido-3,6,9,12-tetraoxapentadec-14-yne (s-2a)** as a clear oil (7.55 g, 86% yield). **IR (thin film/NaCl)** 3252 (w), 2869 (s), 2110 (s), 1460 (w), 1349 (m), 1103 (s), 943 (w), 848 (w), 663 (w) cm^{-1}; **^{1}HNMR (400 MHz, CDCl$_3$)** δ 4.13-4.23 (d, *J* = 2.4 Hz, 2H), 3.71 – 3.59 (m, 14H), 3.41 – 3.30 (t, *J* = 5.1 Hz, 2H), 2.41 (t, *J* = 2.4 Hz, 1H); **^{13}CNMR (125 MHz, CDCl$_3$)** δ 79.55, 74.47, 70.47, 70.45, 70.43, 70.42, 70.20, 69.84, 68.91, 58.18, 50.50; **HRMS (ES+)** calc'd for $C_{11}H_{19}N_3O_4$ (M+H) *m/z* 258.14483. Found 258.14496.

3,6,9,12-tetraoxapentadec-14-yn-1-amine (s-3a)


s-3a

1-azido-3,6,9,12-tetraoxapentadec-14-yne (s-2a) (4.0 g, 15.5 mmol, 1 equiv.), triphenylphosphine (4.87 g, 18.6 mmol, 1.2 equiv.), and water (279 μL, 15.5 mmol, 1.0 equiv.) were dissolved in THF (40 mL) and stirred for 12 h. The reaction was then concentrated and chromatographed (3 cm x 20 cm silica, CH_2Cl_2 then ramp to 80:20:1 CH_2Cl_2:MeOH:Et_3N) to yield **3,6,9,12-tetraoxapentadec-14-yn-1-amine (s-3a)** as a clear oil (2.017 g, 56% yield). **IR (thin film, NaCl)** 3372 (br), 3251 (s), 2868 (s), 2112 (w), 1652 (m), 1596 (m), 1459 (m), 1350 (m), 1301 (m), 1249 (m), 1100 (s), 946 (m), 681 (m) cm^{-1}. **^{1}H-NMR (500 MHz, CDCl$_3$)** δ 4.16 (m, 2H), 3.66-3.57 (m, 12H), 3.49 (t, *J*= 5.3 Hz, 2H), 2.85 (t, *J* = 5.0 Hz, 2H), 2.56 (bs, 2H), 2.41 (m, 1H). **^{13}C NMR (125 MHz, CDCl$_3$)** δ 79.52, 74.63, 73.06, 70.45, 70.41, 70.24, 70.11, 68.96, 58.26, 41.50. **HRMS (ES+)** calc'd for $C_{11}H_{21}NO_4$ (M+H) *m/z* 232.154335. Found 232.15402.

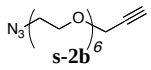
N-(2,4-dinitrophenyl)-3,6,9,12-tetraoxapentadec-14-yn-1-amine (s-4a)


s-4a

3,6,9,12-tetraoxapentadec-14-yn-1-amine (s-3a) (1.0 g, 4.3 mmol, 1 equiv.) was dissolved in EtOH (18mL), and triethylamine (1.68mL, 8.6mmol, 2 equiv.) and 1-chloro-2,4-dinitrobenzene (876 mg, 4.3 mmol, 1 equiv.) were then added. The reaction flask was fitted with a reflux condenser, and the reaction was heated to reflux for 2 h, cooled, and concentrated to afford a crude yellow oil. The crude mixture was re-dissolved in H_2O (25 mL) and

extracted with CH_2Cl_2 (5 x 10 mL). Organic layers were dried over Na_2SO_4, concentrated *in vacuo*, and chromatographed (silica gel, 25g RediSep pre-packed column, 10% EtOAc:Hexanes→ 50% EtOAc:Hexanes, followed by EtOAc flush) to yield **N-(2,4-dinitrophenyl)-3,6,9,12-tetraoxapentadec-14-yn-1-amine (s-4a)** as a yellow solid (1.70g, >98% yield). **IR (thin film/NaCl)** 3360 (m), 3290 (m), 3110 (w), 2873 (m), 2114 (w), 1621 (s), 1588 (m), 1336 (s), 1134 (m) cm^{-1}; **^{1}H-NMR (500 MHz, $CDCl_3$)** δ 9.13 (d, 1H, *J* = 2.6 Hz), 8.80 (bs, 1H), 8.25 (dd, *J* = 2.6, *J*= 9.5 Hz, 2H), 6.94 (d, 1H, *J* = 9.5 Hz), 4.18 (m, 2H), 3.83 (t, *J* = 5.2 Hz, 2H), 3.67 (m, 12H), 3.60 (q, 2H), 2.41 (m, 1H); **^{13}C-NMR (125 MHz, $CDCl_3$)** δ 148.6, 136.2, 130.4, 124.4, 114.3, 79.8, 74.7, 70.8, 70.7, 70.5, 69.2, 68.7, 58.5, 43.4; **HRMS (EI)** calc'd for $C_{17}H_{23}N_3O_8$ (MH+) *m/z* 398.1558. Found 398.1557.

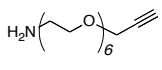
1-azido-3,6,9,12,15,18-hexaoxahenicos-20-yne (s-2b)



s-2b

23-Azido-3,6,9,12,15,-heptaoxatricosan-1-ol (s-1a) (3.3g, 10.7 mmol, 1.0 equiv.) was dissolved in N,N'-dimethylformamide (18 mL) and the resulting solution was cooled to 0 °C. Sodium hydride (516 mg, 21.5 mmol, 2.0 equiv.) was added slowly, followed by propargyl bromide (80% in PhMe, 2.3 mL, 21.5 mmol, 2.0 equiv.), which was added dropwise. The reaction was allowed to stir for 1 h at rt, at which time it was found complete by NMR analysis. The reaction mixture was taken up in CH_2Cl_2 (25 mL) and washed with a saturated aqueous ammonium chloride solution (25 mL). The aqueous solution was back-extracted with dichloromethane (2 x 10 mL), and the combined organics were dried over $MgSO_4$ and concentrated to provide a brown oil. Chromatography (3 cm x 20 cm silica gel, 3% MeOH/ CH_2Cl_2) yielded **1-azido-3,6,9,12,15,18-hexaoxahenicos-20-yne (s-3b)** (2.0g, 61% yield). **IR (thin film)** 2867 (m), 2107 (m), 11349 (s), 1093 (s) cm^{-1}; **^{1}HNMR (400 MHz, $CDCl_3$)** δ 4.20 (d, *J* = 2.4 Hz, 2H), 3.58 (m, 22H), 3.39 (t, *J* = 5.1 Hz, 2H), 2.43 (t, *J* = 2.4 Hz, 1H); **^{13}CNMR (125 MHz, $CDCl_3$)** δ 80.06, 74.58, 70.66, 70.61, 70.43, 69.13, 58.41, 50.70; **HRMS (ES+)** calc'd for $C_{15}H_{27}N_3O_6$ (M+Na) *m/z* 368.17921. Found 368.17925.

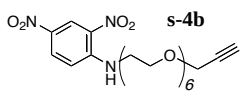
3,6,9,12,15,18-hexaoxahenicos-20-yn-1-amine (s-3b)



s-3b

1-azido-3,6,9,12,15,18-hexaoxahenicos-20-yne (s-2b) (1 g, 2.8 mmol, 1 equiv.), triphenylphosphine (1.1 g, 4.2 mmol, 1.5 equiv.), and water (75 μL, 4.2 mmol, 1.5 equiv.) were dissolved in THF (40 mL) and stirred for 12 h. The reaction mixture was then concentrated, chromatographed (3 cm x 20 cm Silica, CH_2Cl_2 then ramp to 80:20:1 CH_2Cl_2:MeOH:Et_3N), and product-containing fractions were concentrated to yield **3,6,9,12,15,18-hexaoxahenicos-20-yn-1-amine (s-3b)** as a clear oil (240 mg, 25% yield). **IR (thin film)** 3236 (br), 2869 (m), 1620 (m), 1084 (s) cm^{-1}. **^{1}H-NMR (500 MHz, $CDCl_3$)** δ 3.95 (d, 2H, *J* = 2.4), 3.55-3.32 (m, 20H), 3.27 (t, *J* = 5.3 Hz, 2H), 2.62 (t, *J* = 5.0 Hz, 2H), 2.31 (t, *J* = 2.4 Hz, 1H), 1.20 (br s, 2H). **^{13}C NMR (125 MHz, $CDCl_3$)** δ 79.26, 74.40, 73.02, 70.14, 70.10, 69.93, 69.83, 68.62, 57.90, 41.38. **HRMS (ES+)** calc'd for $C_{15}H_{29}NO_6$ (M+H) *m/z* 320.20676. Found 320.20573.

N-(2,4-dinitrophenyl)-3,6,9,12,15,18, -octaoxaheptacos-20-yn-1-amine (s-4b)

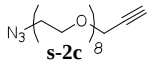


s-4b

3,6,9,12,15,18-hexaoxahenicos-20-yn-1-amine (s-3b) (240 mg, 0.75 mmol, 1 equiv.) was dissolved in EtOH (3 mL). Triethylamine (220 μL, 1.125 mmol, 1.5

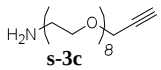
equiv.) and 1-chloro-2,4-dinitrobenzene (167 mg, 0.83 mmol, 1.1 equiv.) were added to the solution. The reaction flask was fitted with a reflux condenser and the reaction solution was heated to reflux for 12 h, cooled, and concentrated to provide a yellow oil. The crude mixture was purified by flash chromatography (3 cm x 20 cm silica gel, 3% MeOH:CH_2Cl_2) to yield **N-(2,4-dinitrophenyl)-3,6,9,12,15,18, -octaoxaheptacos-20-yn-1-amine (s-4b)** as a yellow oil (300 mg, 83% yield). **IR (thin film)** 3360 (w), 2868 (s), 1620 (s), 1334 (m), 1097(s) cm^{-1}; **^{1}H-NMR (500 MHz, $CDCl_3$)** δ 8.94 (d, 1H, *J* = 2.6 Hz), 8.69 (bs, 1H), 8.11 (dd, 1H, *J* = 2.6, *J*= 9.5 Hz), 6.94 (d, 1H, *J* = 9.5 Hz), 4.06 (t, *J* = 2.4 Hz, 2H), 3.74 (t, *J* = 5.0 Hz, 2H), 3.64 (m, 22H), 2.37 (t, 2.38 Hz, 1H); **^{13}C-NMR (125 MHz, $CDCl_3$)** δ 148.2, 135.6, 130.28, 129.9, 123.8, 114.2, 79.5, 70.3, 70.2, 68.3, 58.1, 43.0; **HRMS (EI)** calc'd for $C_{21}H_{31}N_3O_{10}$ (M+H) *m/z* 486.208221. Found 486.20822.

1-azido-3,6,9,12,15,18,21,24-octaoxaheptacos-26-yne (s-2c)


s-2c

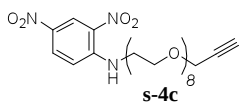
23-Azido-3,6,9,12,15,18,21-heptaoxatricosan-1-ol (s-1c) (1.1g, 3.17 mmol, 1.0 equiv.) was dissolved in N,N'-dimethylformamide (6 mL). To this solution was added sodium hydride (152 mg, 6.34 mmol, 2.0 equiv.), followed by propargyl bromide (80% in PhMe, 683 μL, 6.34 mmol, 2.0 equiv.). The reaction was allowed to stir for 4 h at rt, at which time it was found complete by NMR aliquot. The reaction was then taken up in CH_2Cl_2 (25 mL) and washed with a saturated aqueous ammonium chloride solution (25 mL). The aqueous solution was back-extracted with dichloromethane (2 x 10 mL), and the combined organics were dried over $MgSO_4$ and concentrated to give a brown oil. Chromatography (3 cm x 20 cm silica gel, 3% MeOH/ CH_2Cl_2) yielded **1-azido-3,6,9,12,15,18,21,24-octaoxaheptacos-26-yne (s-2c)** (960 mg, 78% yield). **IR (thin film/NaCl)** 2874 (m), 2110 (m), 1160 (s), 1105 (s) cm^{-1}; **^{1}HNMR (400 MHz, $CDCl_3$)** δ 4.20 (d, *J* = 2.4 Hz, 2H), 3.58 (m, 30H), 3.39 (t, *J* = 5.1 Hz, 2H), 2.43 (t, *J* = 2.4 Hz, 1H); **^{13}CNMR (125 MHz, $CDCl_3$)** δ 79.82, 74.72, 70.75, 7022, 68.27, 58.62, 50.84; **HRMS (ES+)** calc'd for $C_{19}H_{35}N_3O_8$ (M+Na) *m/z* 456.231637. Found 456.23182.

3,6,9,12,15,18,21,24-octaoxaheptacos-26-yn-1-amine (s-3c)


s-3c

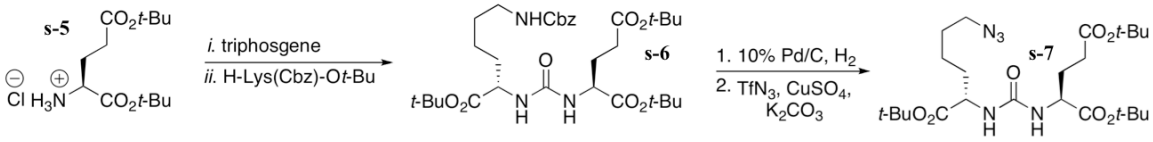
1-azido-3,6,9,12,15,18,21,24-octaoxaheptacos-26-yne (s-2c) (960 mg, 2.52 mmol, 1 equiv.), triphenylphosphine (992 mg, 3.78 mmol, 1.5 equiv.), and water (68 μL, 3.78 mmol, 1.5 equiv.) were dissolved in THF (10 mL) and allowed to stir for 12 h. At that time the reaction was concentrated, chromatographed (3 cm x 20 cm Silica, CH_2Cl_2 then 80:20:1 CH_2Cl_2:MeOH:Et_3N), and concentrated to yield **3,6,9,12,15,18,21,24-octaoxaheptacos-26-yn-1-amine (s-3c)** as a clear oil (815 mg, 91% yield). **IR (thin film, NaCl)** 3105 (br), 2914 (m), 1781 (m), 1638 (m), 1169 (s) cm^{-1}; **^{1}H-NMR (500 MHz, $CDCl_3$)** δ 4.17 (d, *J* = 2.4, 2H), 3.66-3.57 (m, 28H), 3.54 (t, *J* = 5.3 Hz, 2H), 2.88 (t, *J* = 5.0 Hz, 2H), 2.41 (t, *J* = 2.4 Hz, 1H), 2.18 (br s, 2H); **^{13}C NMR (125 MHz, $CDCl_3$)** δ 79.52, 74.59, 72.54, 70.41, 70.38, 70.38, 70.35, 70.31, 70.20, 70.06, 68.90, 58.20, 41.44; **HRMS (ES+)** calc'd for $C_{19}H_{37}NO_8$ (M+H) *m/z* 408.259194. Found 408.25712.

N-(2,4-dinitrophenyl)-3,6,9,12,15,18,21,24-octaoxaheptacos-26-yn-1-amine (s-4c)


s-4c

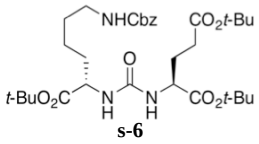
3,6,9,12,15,18,21,24-octaoxaheptacos-26-yn-1-amine (s-3c) (815 mg, 2.27 mmol, 1 equiv.) was dissolved in EtOH (10 mL), and to this solution was added

triethylamine (666 μL, 0.726 mmol, 1.5 equiv.) and 1-chloro-2,4-dinitrobenzene (505 mg, 2.5 mmol, 1.5 equiv.). The reaction flask was fitted with a reflux condenser and the mixture was heated to reflux for 48 h, cooled, and concentrated to provide a yellow oil. The crude mixture was purified by flash chromatography (3 cm x 20 cm silica gel, 3% MeOH:CH_2Cl_2) to yield **N-(2,4-dinitrophenyl)-3,6,9,12,15,18,21,24-octaoxaheptacos-26-yn-1-amine (s-4c)** as a yellow solid (1.15 g, >95% yield). **IR (thin film/NaCl)** 3363 (w), 2871 (s), 1621 (s), 1337 (m), 1103 (s) cm^{-1}; **^{1}H-NMR (500 MHz, $CDCl_3$)** δ 9.08 (d, *J* = 2.6 Hz, 1H), 8.77 (bs, 1 H), 8.21 (dd, *J* = 2.6, *J*= 9.5 Hz, 1H), 6.94 (d, *J* = 9.5 Hz, 1H), 4.16 (6, *J* = 2.4 Hz, 2H), 3.78 (t, *J* = 5.0 Hz, 2H), 3.64 (m, 28H), 3.58 (q, 2H), 2.41 (t, 2.38 Hz, 1H); **^{13}C-NMR (125 MHz, $CDCl_3$)** δ 148.5, 136.1, 130.5, 130.29, 124.3, 114.3, 79.8, 74.6, 70.7, 70.6, 70.5, 69.2, 68.7, 58.5, 43.3; **HRMS (EI)** calc'd for $C_{25}H_{39}N_3O_{12}$ (M+H) *m/z* 574.260650. Found 574.26106.



Scheme s2. Synthesis of **s-7**.

(9S,13S)-tri-tert-butyl 3,11-dioxo-1-phenyl-2-oxa-4,10,12-triazapentadecane-9,13,15-tricarboxylate (s-6)



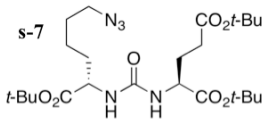
L-glutamic acid di-tertbutyl ester hydrochloride (s-5) (1.0g, 3.38 mmol, 1.0 equiv.) and triethylamine (1.54 mL, 11.09 mmol, 3.28 equiv.) were dissolved in CH_2Cl_2 (30 mL) and the resulting solution was cooled to -78 ^oC. Triphosgene (341 mg, 1.15 mmol, 0.34 equiv.) in CH_2Cl_2 (10 mL) was added dropwise via syringe to the reaction mixture. Upon complete addition, the reaction was allowed to warm to room temperature and stir for 30 minutes. H-Lys(Z)-O*t*-Bu Hydrochloride (757 mg, 2.03 mmol, 0.6 equiv) was added, followed by triethylamine (283 μL, 2.03 mmol, 0.6 equiv.). The reaction was allowed to stir at room temperature overnight for 16 h. The reaction was then diluted with CH_2Cl_2 (50 mL), and washed with H_2O (2 x 100 mL). The crude mixture was dried over Na_2SO_4 and concentrated under reduced pressure. Column chromatography of the resulting oil (3 x 25 cm silica gel, 1.5:1 hexane:EtOAc) yielded **s-6** as a colorless oil (1.09g, 86%). **IR (thin film/KBr)** 3342 (br), 2976 (m), 1731 (s), 1650 (m), 1552 (s), 1454 (w), 1368 (m), 1255 (s), and 1153 (s) cm^{-1}; **^{1}H NMR (500 MHz, $CDCl_3$)** δ 7.35 (d, *J* = 3.8 Hz, 4H), 7.33-7.30 (m, 1H), 5.10 (d, *J* = 4.6 Hz, 2H), 5.06-5.01 (m, 2H), 4.99 (s, 1H), 4.34-4.31 (m, 2H), 3.20-3.18 (m, 2H), 2.36-2.23 (m, 2H), 2.10-2.03 (m, 1H), 1.88-1.75 (m, 2H), 1.65-1.57 (m, 1H), 1.57-1.45 (m, 2H), 1.453 (s, 9H), 1.446 (s, 9H), 1.43 (s, 9H), 1.40-1.30 (m, 2H); **^{13}C NMR (125 MHz, $CDCl_3$)** δ 172.6, 172.5, 172.2, 136.8, 128.6, 128.5, 128.2, 82.2, 82.0, 80.7, 66.7, 53.4, 53.2, 40.7, 32.8, 31.7, 29.4, 28.5, 28.2, 28.1, 22.3; **HRMS (EI)** calc'd for $C_{25}H_{39}N_3O_{12}$ (M+H) *m/z* 622.3698. Found 622.3695.

(S)-di-tert-butyl 2-(3-((S)-6-amino-1-tert-butoxy-1-oxohexan-2-yl)ureido)pentanedioate (s-6b)

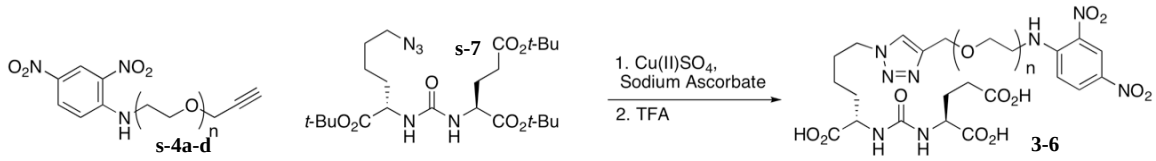
s-6 (2.35 g, 3.78 mmol, 1.0 equiv.) was dissolved in MeOH (40 ml) and added dropwise to a vigorously stirred reaction flask containing dry 10% Pd/C (475 mg, 20% by mass). H_2 was introduced via cannula and

allowed to bubble through the reaction solution for 1-2 m, and then the mixture was allowed to stir for 13 h under a balloon of H_2. The reaction was deemed complete by TLC (R_f = 0.48 in 10% MeOH/CH_2Cl_2**)**, filtered through a plug of celite, and concentrated to give a viscous oil, which was used in the next step without further purification.

(S)-di-tert-butyl 2-(3-((S)-6-azido-1-tert-butoxy-1-oxohexan-2-yl)ureido)pentanedioate (s-7)

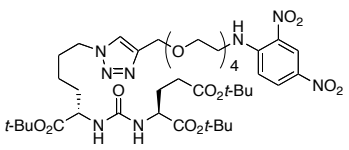


Sodium azide (2.629 g, 40.75 mmol, 10.0 equiv.) was dissolved in H_2O (8 mL), and CH_2Cl_2 (13 mL) was added. The reaction mixture was cooled to 0 ^oC and triflic anhydride (1.4 mL, 8.09 mmol, 2.0 equiv.) was added. The solution was stirred for 3 h at rt, and the organic layer was separated from the aqueous layer. The aqueous layer was extracted with CH_2Cl_2 (3 x 4 mL). The organic layers were combined and washed with saturated aqueous Na_2CO_3 (10 mL) to provide 25 ml of a solution assumed to contain TfN_3 (theoretical concentration of 0.391 M). In a separate flask, **s-6b** (1.97 g, 4.04 mmol, 1.0 equiv.) was dissolved in H_2O (14 mL) and MeOH (29 mL). To this solution were added $CuSO_4$-$5H_2O$ (10.1 mg, 0.04 mmol, 0.01 equiv.) and K_2CO_3 (837.5 mg, 6.06 mmol, 1.5 equiv.). The TfN_3 solution (25 ml, 8.09 mmol, 2 equiv.) was added rapidly to the stirring solution of **s-6b**, and the reaction was allowed to stir for 19 h at rt. The organic layer was separated from the aqueous layer, and the H_2O/MeOH layer was extracted once with dichloromethane. The combined organic layers were dried over $MgSO_4$, concentrated under reduced pressure, and purified by column chromatography (3 x 25 cm silica gel, 10% MeOH in CH_2Cl_2) to yield **s-7** as a white solid (1.440 g, 71%). R_f =0.68 in 10% MeOH:CH_2Cl_2. **IR (Thin film / NaCl)** 3335 (br), 2980 (m), 2933 (m), 2868 (w), 2097 (s), 1733 (s), 1635 (s), 1560 (m), 1368 (s), 1257 (m), and 1155 (s) cm^{-1}; **^{1}HNMR (500 MHz, $CDCl_3$)** δ 5.01 (d, *J* = 8.25 Hz, 2H), 4.34 (m, 2H), 3.26 (t, *J* = 7.4 Hz, 2H), 2.35-2.25 (m, 2H), 2.09-2.05 (m, 1H), 1.87-1.76 (m, 2H), 1.66-1.55 (m, 3H), 1.46 (s, 18H), 1.43 (s, 9H), 1.45-1.35 (m, 2H) ppm; **^{13}CNMR (125 MHz, $CDCl_3$)** δ 172.6, 172.4, 172.2, 156.8, 82.3, 82.1, 80.7, 53.4, 53.2, 51.3, 33.0, 31.7, 28.6, 28.5, 28.2, 28.1, 22.4 ppm; **HRMS (EI)** calc'd for $C_{25}H_{39}N_3O_{12}$ (M+H) *m/z* 514.3235. Found 514.3225.



Scheme s3. General synthesis of ARM-Ps **3-6**. **a**, n=4; **b**, n=6; **c**, n=8; **d**, n=12.

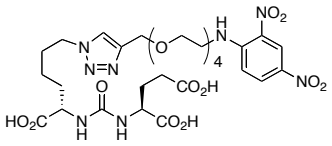
(S)-di-tert-butyl 2-(3-((S)-1-tert-butoxy-6-(4-(13-(2,4-dinitrophenylamino)-2,5,8,11-tetraoxatridecyl)-1H-1,2,3-triazol-1-yl)-1-oxohexan-2-yl)ureido)pentanedioate (s-8)



A mixture of **s-7** (103.3 mg, 0.20 mmol, 1.0 equiv.) and **s-4a** (88.1 mg, 0.22 mmol, 1.1 equiv.) was dissolved in a mixture of H_2O (0.694 mL) and *tert*-butanol (0.694 mL) in a 5 ml μwave reaction tube. To this solution was added 0.1 M sodium ascorbate (0.388 ml, 0.0388 mmol, 0.19 equiv.) and 0.1 M copper (II) sulfate (0.078 ml, 0.0078 mmol, 0.04 equiv.). The tube was capped, and

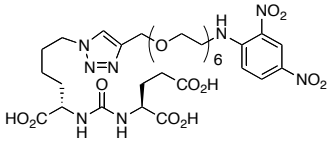
subjected to μwave irradiation for 10 minutes at 110°C. The reaction was then concentrated and chromatographed (1.5 x 15 cm silica gel, 5% methanol in dichloromethane) to obtain a partially purified material, which was further chromatographed (2% MeOH in CH_2Cl_2 to 5% methanol in dichloromethane) to yield **s8** (129.9 mg, 71%) as a brown oil. **IR (thin film, NaCl)** 3949 (br), 2976 (m), 2932 (m), 2872 (m), 1734.5 (m), 1623 (m), 1368 (m), 1153 (m) cm^{-1}; **^{1}HNMR (400 MHz, $CDCl_3$)** δ ppm 9.14 (d, *J* = 2.68 Hz, 1H) 8.81 (s, 1H), 8.26 (dd, *J* = 2.68, 9.5 Hz, 1H), 7.62 (s, 1H), 6.96 (d, *J* = 9.6 Hz, 1H), 5.15 (d, *J* = 7.92 Hz, 1H), 5.05 (d, = 7.64 Hz, 1H), 4.71-4.63 (dd, *J* = 12.4, 20.32 Hz, 2H), 4.36-4.29 (m, 4H), 3.83 (t, *J* = 5.24 Hz, 2H), 3.71-3.65 (m, 12H), 3.62-3.58 (q, *J* = 5.24, 2H), 2.38-2.24 (m, 2H), 2.11-2.01 (m, 1H), 1.96-1.75 (m, 4H), 1.61-1.56 (m, 1H), 1.46 (s, 9H), 1.43 (s, 18H), 1.38-1.25 (m, 2H) ppm; **^{13}CNMR (125 MHz, $CDCl_3$)** δ 172.6, 172.2, 172.2, 156.9, 148.6, 145.2, 136.2, 130.7, 130.4, 124.5, 123.0, 114.3, 82.1, 82.1, 80.7, 77.7, 70.9, 70.8, 70.8, 70.7, 69.7, 68.8, 64.7, 53.3, 50.0, 43.4, 32.6, 31.8, 29.7, 28.4, 28.2, 28.2, 28.1, 21.9 ppm; **HRMS (EI+)** calc'd for $C_{41}H_{66}N_8O_{15}$ (M+H) *m/z* 911.4720. Found 911.4730.

(S)-2-(3-((S)-1-carboxy-5-(4-(13-(2,4-dinitrophenylamino)-2,5,8,11-tetraoxatridecyl)-1H-1,2,3-triazol-1-yl)pentyl)ureido)pentanedioic acid (3, ARM-P4)



s-8 (59.1 mg, 0.065 mmol) was dissolved in a mixture of trifluoroacetic acid (2 mL) and CH_2Cl_2 (1 mL) in a 5 ml μwave reaction tube. The tube was capped and subjected to microwave irradiation for 2 m at 70 °C. The resulting reaction mixture was concentrated under reduced pressure to yield **ARM-P4 (3)** (51.3 mg, >98% yield) as a yellow oil without need for further purification. **IR (thin film, NaCl)** 3949 (br), 2874 (m), 1730 (m), 1621(m), 1336 (m), 1089 (m) cm^{-1}; **^{1}HNMR (125 MHz, MeOD)** δ 9.00 (d, J = 2.65 Hz, 1H), 8.26 (dd, J = 2.65, 9.6 Hz, 1H), 8.03 (s, 1H), 7.19 (d, J = 9.6 Hz, 1H), 4.63 (s, 2H), 4.42 (t, J = 7 Hz, 2H), 4.31-4.26 (m, 2H), 3.79 (t, J = 7 Hz, 2H), 3.68-3.59 (m, 14H), 2.44-2.36 (m, 2H), 2.17-2.08 (m, 1H), 1.99-1.82 (m, 4H), 1.71-1.64 (m, 1H), 1.46-1.38 (m, 2H); **^{13}CNMR (125 MHz, DMSO)** δ 176.4, 176.1, 175.8, 160.0, 149.9, 137.0, 131.6, 131.0, 125.4, 124.7, 116.1, 71.6, 71.6, 71.5, 71.5, 70.9, 69.9, 64.8, 53.7, 53.5, 51.2, 44.1, 32.8, 31.1, 30.6, 28.8, 23.4 ppm; **HRMS (EI+)** calc'd for $C_{29}H_{42}N_8O_{15}$ (M+H) *m/z* 743.2843. Found 743.2850.

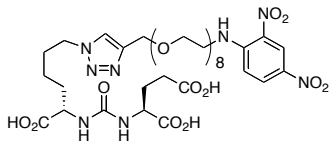
(S)-2-(3-((S)-1-carboxy-5-(4-(19-(2,4-dinitrophenylamino)-2,5,8,11,14,17-hexaoxanonadecyl)-1H-1,2,3-triazol-1-yl)pentyl)ureido)pentanedioic acid (4, ARM-P6)



A mixture of **s-4b** (90 mg, 0.185 mmol, 1.0 equiv.) and **s-7** (95 mg, 0.185 mmol, 1.0 equiv.) was transferred to a 5 ml μwave reaction tube and dissolved in a mixture of H_2O (1 mL) and *tert*-butanol (1 mL). To this was added sodium ascorbate (9 mg, 0.046 mmol, 0.25 equiv.) and an aqueous solution of 0.1 M copper (II) sulfate (0.0925 ml, 0.00925 mmol, 0.05 equiv.). The tube was capped, and subjected to μwave irradiation for 10 minutes at 110°C. The reaction was then concentrated and redissolved in trifluoroacetic acid (2 mL) and CH_2Cl_2 (1 mL) in a 5 ml μwave reaction tube. The tube was capped and subjected to microwave irradiation for 2 m at 70°C. The resulting reaction mixture was concentrated *in vacuo*, chromatographed using HPLC (Sunfire™ Prep C18 column (10 x 150 mm) using a H_2O to 65% AcCN in H_2O gradient over

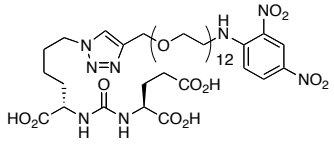
36 min at 5 mL/min), and concentrated to yield **ARM-P6 (4)** (70 mg, 45% yield) as a yellow oil. **IR (thin film)** 3361 (w), 2925 (s), 1722 (s), 1619 (m), 1086 (s) cm^{-1}1H NMR **(500 MHz, MeOH)** δ 9.04 (d, J = 2.7 Hz, 1H), 8.29 (dd, J = 2.7, 9.6 Hz, 1H), 8.00 (s, 1H), 7.23 (d, J = 9.6 Hz, 1H), 4.63 (s, 2H), 4.42 (t, J = 6.9 Hz, 2H), 4.36 – 4.21 (m, 2H), 3.81 (t, J = 5.2 Hz, 2H), 3.72 – 3.55 (m, 21H), 2.42 (dd, J = 4.9, 9.1 Hz, 2H), 2.15 (d, J = 7.2 Hz, 1H), 2.05 – 1.78 (m, 5H), 1.70 (s, 1H), 1.42 (d, J = 7.4 Hz, 2H).; ^{13}C NMR (125 MHz, MeOH) δ 176.4, 176.1, 175.8, 160.1, 149.9, 146.0, 137.1, 131.6, 131.0, 125.2, 124.7, 116.2, 71.6, 71.6, 71.6, 71.6, 71.6, 71.5, 70.8, 70.0, 65.0, 53.7, 53.5, 51.2, 44.1, 32.9, 31.1, 30.8, 28.9, 23.5 ppm; **HRMS (ES+)** calc'd for $C_{33}H_{50}N_8O_{17}$ (M+H) *m/z* 831.33667. Found 831.33595.

(S)-2-(3-((S)-1-carboxy-5-(4-(25-(2,4-dinitrophenylamino)-2,5,8,11,14,17,20,23-octaoxapentacosyl)-1H-1,2,3-triazol-1-yl)pentyl)ureido)pentanedioic acid (5, ARM-P8)



To a mixture of **s-4c** (76 mg, 0.145 mmol, 1.0 equiv.) and **s-7** (74.4 mg, 0.145 mmol, 1.0 equiv.) in H_2O (1 mL) and *tert*-butanol (1 mL) in a 5 ml μwave reaction tube was added sodium ascorbate (7 mg, 0.036 mmol, 0.25 equiv.) and an aqueous solution of 0.1 M copper (II) sulfate (0.0725 ml, 0.00725 mmol, 0.05 equiv.). The tube was capped, and subjected to μwave irradiation for 10 minutes at 110°C. The reaction was then concentrated and redissolved in trifluoroacetic acid (2 mL) and CH_2Cl_2 (1 mL) in a 5 ml μwave reaction tube. The tube was capped and subjected to microwave irradiation for 2 m at 70 °C. The resulting reaction mixture was concentrated under reduced pressure, chromatographed using HPLC (See ARM-P6 purification), and concentrated to yield **ARM-P8 (5)** (87 mg, 58% yield) as a yellow oil. **IR (thin film/NaCl)** 3359 (w), 2925 (s), 1737 (s), 1622 (m), 1170 (s) cm^{-1}; **^{1}HNMR (100 MHz, MeOD)** δ 9.07 (d, J = 2.7 Hz, 1H), 8.33 (dd, J = 2.7, 9.6 Hz, 1H), 8.03 (s, 1H), 7.27 (d, J = 9.6 Hz, 1H), 4.66 (s, 2H), 4.45 (t, *J* = 7 Hz, 2H), 4.35-4.28 (m, 2H), 3.83 (t, J = 7 Hz, 2H), 3.73-3.61 (m, 30H), 2.44-2.36 (m, 2H), 2.17-2.08 (m, 1H), 1.99-1.82 (m, 4H), 1.71-1.64 (m, 1H), 1.46-1.38 (m, 2H); **^{13}CNMR (125 MHz, MeOD)** δ 176.4, 176.1, 175.7, 160.0, 149.9, 145.9, 137.0, 131.5, 131.0, 125.2, 124.7, 116.1, 71.6, 71.6, 71.5, 71.5, 70.9, 69.9, 64.8, 53.7, 53.5, 51.2, 44.1, 32.8, 31.1, 30.6, 28.8, 23.4 ppm; **HRMS (ES+)** calc'd for $C_{37}H_{58}N_8O_{19}$ (M+H) *m/z* 919.389098. Found 919.38801.

(S)-2-(3-((S)-1-carboxy-5-(4-(37-(2,4-dinitrophenylamino)-2,5,8,11,14,17,20,23,26,29,32,35-dodecaoxaheptatriacontyl)-1H-1,2,3-triazol-1-yl)pentyl)ureido)pentanedioic acid (6, ARM-P12)



In a 5 ml μwave reaction tube, **DNP-NH(C_2H_2O)$_{12}$$CH_2$CCH (s-4d)**[8] (48 mg, 0.063 mmol, 1.0 equiv.) and **s-7** (32.7 mg, 0.063 mmol, 1.0 equiv.) were dissolved in H_2O (0.5 mL) and *tert*-butanol (0.5 mL). To this solution was added sodium ascorbate (3 mg, 0.0157 mmol, 0.25 equiv.) and an aqueous solution of 0.1 M copper (II) sulfate (0.032 ml, 0.00315 mmol, 0.05 equiv.). The tube was capped, and subjected to μwave irradiation for 10 minutes at 110°C. The reaction was then concentrated and redissolved in trifluoroacetic acid (2 mL) and CH_2Cl_2 (1 mL) in a 5

[8] **s-4d** was obtained in 6% yield from the starting dodecaethylene glycol following procedures used for other oligomers.

ml μwave reaction tube. The tube was capped and subjected to microwave irradiation for 2 m at 70 ^{o}C. The resulting reaction mixture was concentrated under reduced pressure, chromatographed using HPLC (See ARM-P6 purification), and concentrated to yield **ARM-P12 (6)** (50 mg, 72% yield) as a yellow oil. **IR (thin film)** 3361 (w), 2870 (s), 1725 (s), 1620 (m), 1089 (s) cm^{-1}; **^{1}HNMR (100 MHz, MeOD)** δ 8.98 (d, *J* = 2.6, 1H), 8.25 (dd, J = 2.6, 9.6, 1H), 7.96 (s, 1H), 7.19 (d, J = 9.6, 1H), 4.59 (s, 2H), 4.38 (t, *J* = 7.0, 2H), 4.25 (s, 2H), 3.83 – 3.71 (m, 6.0, 48H), 2.38 (s, 2H), 2.11 (s, 1H), 1.99 – 1.72 (m, 4H), 1.65 (d, *J* = 7.7, 1H), 1.42 – 1.25 (m, 2H); **^{13}CNMR (125 MHz, DMSO)** δ 174.4, 174.1, 173.7, 157.3, 148.4, 143.9, 134.9, 129.9, 129.7, 123.7, 123.6, 115.7, 69.8, 69.8, 69.7, 68.9, 68.3, 63.5, 49.1, 42.7, 31.5, 29.9, 29.4, 27.5, 22.1 ppm; **HRMS (ES+)** calc'd for $C_{45}H_{74}N_8O_{23}$ (M+H) *m/z* 1095.4940. Found 1095.4898.

Biology

NAALADase Inhibition Experiments

A10 mM stock solution of N-acetyl-aspartyl-glutamate (NAAG) in 40 mM NaOH was diluted to 40 μM in Tris buffer (0.1M Tris-HCl, pH=7.5), and was added to a 384 well plate (25 μl per well). For K_m measurements and K_m negative controls (absence of PSMA), a 2x dilution (40 μM – 312 nM) series of NAAG were made and added to separate wells. For IC_{50} measurements, solutions of inhibitors in water (2 μL per well, dilution series) were added to wells. For all other wells, 2 μL of water was added to control for volume changes. To initiate reactions, rhPSMA (R&D research) diluted in TBS-A (20 ng/ mL), was added to each well (25 μl per well). For negative controls, TBS-A was added (25 μl per well). The plate was then covered and incubated for 15 m, at which time the protein was deactivated by heating the plate to 95°C for 3 minutes using a sand bath. After the plate was allowed to cool, glutamic acid production was visualized using an Amplex®-Red glutamic acid/glutamate oxidase assay kit (Invitrogen). The plate was read on a Synergy 2 (Biotek) plate-reader with emission and excitation filters of 545/40 and 590/35 respectively. K_m and IC_{50} values were calculated using graphpad prism software, and the reported K_i was calculated from these values using the Cheng-Prusoff equation. This process was run in triplicate, and is reported in the manuscript as the average of three runs ± standard deviation. **Note**: Due to inter-assay variability in the activity of PSMA, prior to running each inhibition experiment, the assay was performed in the absence of inhibitors to determine an enzyme concentration at which conversion of NAAG substrate was held below 15% during the assay.[9]

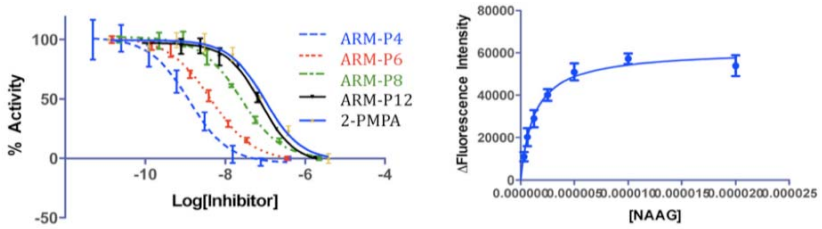


Figure S2. Representative example of inhibition and K_M curves obtained through above experiment.

Trial	n=4 IC_{50} (K_i)	n=6 IC_{50} (K_i)	n=8 IC_{50} (K_i)	n=12 IC_{50} (K_i)	PMPA IC_{50} (K_i)	(K_M)
1	2.3 x 10^{-9} M (8.7 x 10^{-11} M)	9.2 x 10^{-9} M (3.6 x 10^{-10} M)	3.2 x 10^{-8} M (1.2 x 10^{-9} M)	1.1 x 10^{-7} M (4.2 x 10^{-9} M)	4.82 x 10^{-8} M (1.9 x 10^{-9} M)	1.3 x 10^{-8} M
2	1.2 x 10^{-9} M (3.5 x 10^{-11} M)	4.4 x 10^{-9} M (1.2 x 10^{-10} M)	2.4 x 10^{-8} M (6.8 x 10^{-10} M)	7.9 x 10^{-8} M (2.2 x 10^{-9} M)	9.9 x 10^{-8} M (2.8 x 10^{-9} M)	5.7 x 10^{-7} M
3	1.1 x 10^{-9} M (4.2 x 10^{-11} M)	3.9 x 10^{-9} M (1.5 x 10^{-10} M)	1.2 x 10-8 M (4.6 x 10^{-10} M)	5.0 x 10^{-8} M (1.9 x 10^{-9} M)	ND	8.0 x 10^{-7} M
Average K_i ± SD	5.4 x 10^{-11} M ±2.8 x 10^{-11} M	2.1 x 10^{-10} M ±1.3 x 10^{-10} M	7.8 x 10^{-10} M ±3.8 x 10^{-10} M	2.7 x 10^{-9} M ±1.3 x 10^{-9} M		

Table S1. Data compiled from three experiments and average of three runs for ARM-P series of molecules. Also shown are duplicate runs of PMPA giving Ki values consistent with a previously reported literature value (1.4 nM).[10]

[9] The authors strongly recommend personally establishing appropriate concentrations of rhPSMA (ie, good signal but <15% NAAG conversion) prior to running experiments.

[10] Kozikowski, A. P.; Zhang, J.; Nan, F.; Petukhov, P. A.; Grajkowska, E.; Wroblewski, J. T.; Yamamoto, T.; Bzdega, T.; Wroblewska, B.; Neale, J. H. *J. Med. Chem.*, **2004**, *47*, 1729-1738.

Flow Cytometry

ARM-P comparison: LNCaP and DU-145 (negative control) cells were detached, counted, washed, and resuspended in TBS-A to a density of 5 x 10^5 cells mL^{-1}, and 0.25 mL of this suspension was added to Eppendorf tubes. Human IgG (2.5 μL of 10 mM in TBS-A, Bethyl Laboratories, Lot # P80-105L-1) was added to cells to block Fc receptors, and cells were then incubated at room temperature for 5 minutes. The cultures were cooled on ice, and solutions of **ARM-Ps** in water (2.5 μL, 1 μM stocks) were added followed immediately by AlexaFluor488 conjugated rabbit anti-dinitrophenyl IgG – fraction KLH (2.5 μL of 2 mg ml^{-1}, Invitrogen, cat # A11097). Experiments were protected from light and incubated on ice for 10 minutes. The cells were diluted with TBS-A (750 μL), spun down at 1000 rpm and washed with TBS-B (1 x 1 mL). The cells were taken up with 0.5 mL of TBS-B and 2 μL of 500 μg mL^{-1} of propidium iodide was added. Samples were analyzed immediately on a FACSCalibur instrument (Becton Dickinson). The data was analyzed on the FL-1 channel using FlowJo software (Tree Star Inc.) after gating for a single population of live cells on the FL-3 channel. An experiment omitting **3** was performed as a control. These experiments were performed on three separate occasions to ensure reproducibility.

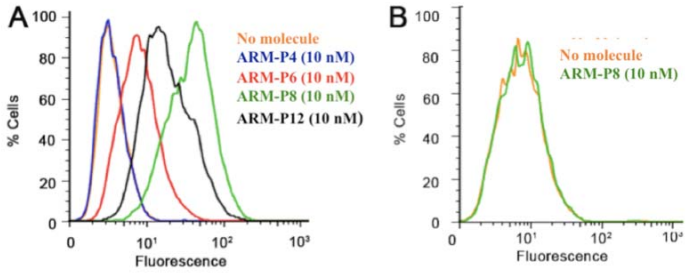


Figure S3: (A) Flow cytometry experiments showing antibody recruitment abilities of ARM-P4, ARM-P6, ARM-P8, and ARM-P12 to PSMA+ LNCaP cells. (B) Flow cytometry experiments showing lack of ARM-P8-mediated anti-DNP binding to PSMA negative DU145 cells .

Di-DNP Lysine and 2-PMPA competition experiments: LNCaP cells were detached, counted, washed, and resuspended in TBS-A to a density of 5 x 10^5 cells mL^{-1} of TBS-A. 0.25 mL of this suspention was then added to each Eppendorf tube for each experiment. Human IgG (2.5 μL of 10 mM in Tris Buffer) was added to cells to block Fc receptors, and cells were then incubated at room temperature for 5 minutes. Solutions of **ARM-Ps** in water (2.5 μL, 5 μM stocks) were added to the cells followed by dilution series of di-DNP Lysine and 2-PMPA (2.5 μL, indicated concentrations), and finally, by AlexaFluor488 conjugated rabbit anti-dinitrophenyl IgG – fraction KLH (2.5 μL of 2 mg ml^{-1}). Experiments were protected from light, incubated at 37 ^oC for 60 minutes, then taken up with 850 μL of flow cytometry buffer. The cells were diluted with TBS-A (750 μL), spun down at 1000 rpm and washed with TBS-A (1 x 1 mL) followed by TBS-B. The cells were suspended in 0.5 mL of TBS-B, 2 μL of 500 μg mL^{-1} of propidium iodide was added, and samples were analyzed immediately on a FACSCalibur flow cytometer (Becton Dickinson). The data was analyzed using FlowJo software (Tree Star Inc.), gating for a single population of live cells on FL-3. An experiment omitting **3** was performed as a control. The experiment was repeated in triplicate

to ensure reproducibility. To determine competition binding constants, fluorescent means were calculated through FlowJo and plotted versus the concentrations and fit to a competition isotherm using graphpad prism:

$$Y=(B*Ct/(B+K1*((X/K3)+1)))$$

where Y = fluorescent mean (variable), X = PMPA concentration (variable), B = concentration of ARM-P8 (10 nM), C_t = concentration of PSMA (estimated at 80 pM), and $K_1 = K_D$ of ARM-P8/Antibody Complex binding to PSMA (estimated to be 780 pM based on prior inhibition experiments). Solved for K_3 (apparent PMPA K_D = 5 nM).

Fluorescent Microscope Experiments

LNCaP cells were trypsynized thoroughly using 0.25% trypsin-EDTA (0.5 mL) and resuspended in growth media to a density of 3 x 10^5 cells mL^{-1} of growth media. To each well of cell culture plates (3x4) containing cover slips and 1 mL of growth media was added 100 μL of cells. Cells were grown on cover slips until they reached 40-60% confluency (approximately 3-4 days). Wells were then washed with TBS-A (2x1 mL), and filled with 500 μL of TBS-A. Human IgG (5 μL of 10 mM in TBS-A) was added to each well to block Fc receptors, and cultures were incubated at room temperature for 5 minutes. One plate was cooled to 4 ^oC prior to further addition of reagents and kept cool on an ice cold metal plate during additions. Solutions of **ARM-P8** in water (5 μL, 5 μM stocks) were added to the cells followed by AlexaFluor488 conjugated rabbit anti-dinitrophenyl IgG – fraction KLH (5 μL of 2 mg ml^{-1}). For negative control experiments (NC), water (5 μL) was added. Cultures were protected from light and incubated at 37^oC (culture oven) or 4 ^oC (refrigerator) for 60 minutes. The wells were washed with TBS-B (2 x 1 mL), fixed by addition of 100 μL of paraformaldehyde (4% in DPBS), and incubated at room temperature for 10 m. Wells were washed with TBS-B (2 x 1 mL) and slides were rinsed with water 5 times before mounting onto glass slides with Gel Mount mounting media (Biomeda Corp.). Cells were analyzed using a Zeiss Axiovert 200M fluorescent microscope equipped with a GFP filter. Experiments were performed 3 times to ensure reproducibility.

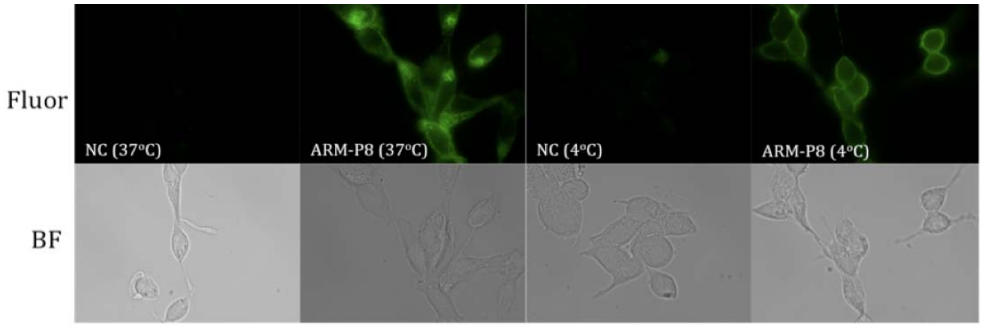


Figure S4: Cellular imaging experiments showing that ARM-P8 (50 nM) is capable of recruiting fluorescently labeled antibodies to LNCaP cells. Internalization of fluorescence is observed at 37^oC but not at 4^oC.

Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC)

Preparation of PBMC: Frozen peripheral blood mononuclear cells (PBMC) were obtained from AllCell, LLC (Cat. No. mPB008F). Frozen PBMC were thawed the day before ADCC experiments. Frozen PBMC were thawed in a 37 ^{o}C water bath and washed twice with 20 mL of RPMI medium 1640 + 10% FBS + 100 U/mL penicillin/streptomycin before use. Washed PBMC were plated at approximately $2.5x10^6$ cells per plate and incubated overnight at 37^{o}C, 5% CO_2. Different lots of PBMC were purchased and screened for ADCC ability before selecting a single lot for all of the reported experiments.

General Procedure for Antibody Dependent Cell-Mediated Cytotoxicity (ADCC) Experiments: Target cells in tissue culture dishes were washed once in ADCC medium and incubated with calcein-AM (10 mL of 10 μM solution in ADCC medium, purchased from Sigma-Aldrich as a 4 mM solution in dry DMSO) for 60 m at 37 ^{o}C, 5% CO_2. After two washes with ADCC medium, cells were detached, diluted to $2.5x10^5$ cells/mL, and plated onto black-plate, clear-bottom 96-well assay plates (Corning, Cat. No. 3603) at $1.25x10^4$ cells/well (50 μL per well). Rabbit anti-dinitrophenyl-KLH IgG (unless otherwise stated 24 μg/mL final well concentration, Invitrogen, Cat. No. A-6430) and ARM-P8 (50 μL, dilution series as indicated) were added into the wells and plates were incubated at 37 ^{o}C, 5% CO_2 for 60 minutes. Following the incubation period, human PBMC (25:1 effecto:target ratio unless otherwise indicated, AllCells, LLC, Cat. No. mPB008F, Lot PCA1258B) were added. Maximum killing was achieved by adding 15 μL of 10% Triton X-100 to 150 μL of cell suspension. To all other wells was added 15 μL of ADCC medium. Controls cultures to determine background release due to non-specific killing consisted of target cells, effector cells, and anti-dinitrophenyl antibodies in the absence of ARM-P8. After incubation at 37^{o} C, 5% CO_2 for 4 h, 50 μL of supernatant was removed and transferred to a new black 96 well plate. Sample fluorescence was analyzed using a Synergy 2 microplate spectrofluorimeter (excitation at 485 +/- 20 nm, emission at 528 +/- 20 nm). Control experiments were performed using PSMA negative DU145 cells as well as using ARM-P4 as the antibody-recruiting molecule. Cytotoxicity experiments were also performed in the absence of antibodies and effector cells, as well as in the absence of effector cells only. % ARM dependent cytotoxicity was calculated using the following formula relative to background killing:

$$\% \text{ Cytotoxicity} = 100*(\text{Fluorescence}_{expt} - \text{Fluorescence}_{\text{no ARM avg}})/(\text{Fluorescence}_{\text{maxkilling avg}} - \text{Fluorescence}_{\text{no ARM avg}})$$

Labeling cells with trinitrophenyl sulfonic acid: Target cells in tissue culture dishes were washed once in ADCC medium and incubated with calcein-AM (10 μM, 10 mL ADCC medium) for 60 minutes at 37^{o}C, 5% CO_2. After two washes with ADCC medium, cells were detached and diluted to $2.5x10^5$ cells/mL in a 15 mL centrifuge tube. Trinitrophenyl sulfonic acid (50 mg/mL in methanol) was added to the cells to

make a final concentration of 100 μg/mL. The cells were incubated at room temperature for 30 minutes, washed three times with ADCC medium, and were immediately used in the above ADCC experiments as a positive control for the anti-DNP antibody and PBMCs.

Procedure for 2-PMPA Competition ADCC Experiments (Figure S5-C): Target cells in tissue culture dishes were washed once in ADCC medium and incubated with calcein-AM (10 μM, 10 mL ADCC medium) for 60 minutes at 37°C, 5% CO_2. After two washes with ADCC medium, cells were detached and diluted to $2.5x10^5$ cells/mL and plated onto black-plate, clear-bottom 96-well assay plates (Corning, Cat. No. 3603) at $1.25x10^4$ cells/well (50 μL per well). Rabbit anti-dinitrophenyl-KLH IgG, ARM-P8 (100 μM) and various concentrations of 2-PMPA (50 μL) were added into the wells, and plates were incubated at 37°C, 5% CO_2 for 60 minutes. Human PBMC (Lot # PCA1319) were then added at an effector:target ratio of 50:1. Maximum killing values were determined by adding 15 μL of 10% Triton X-100 to 150 μL of cell suspension. A volume of ADCC medium (15 μL) was added to all other wells. After incubation at 37°C, 5% CO_2 for 4 h, 50 μL of supernatant was removed and transferred to a new black 96 well plate. Sample fluorescence was measured using a Synergy 2 microplate spectrofluorimeter (excitation at 485 +/- 20 nm, emission at 528 +/- 20 nm).

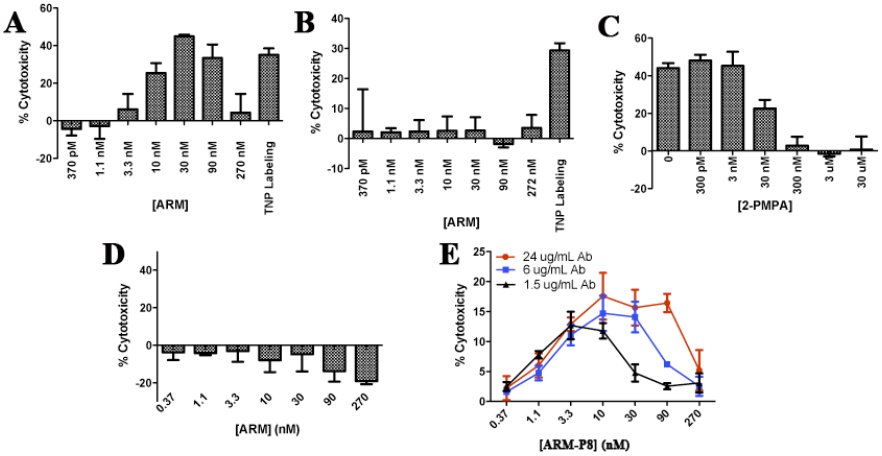
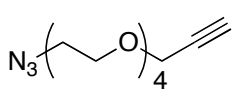


Figure S5: ADCC experiments. (A) ADCC assay results for LNCaP cells in the presence of ARM-P8, anti-DNP antibody, and PBMC compared to ADCC assay results for TNP-labeled cells in the presence of anti-DNP antibody, and PBMC. Notably, maximum values for %cytotoxicity were comparable between these two groups. (B) ADCC assay results for untreated versus TNP-labeled DU145 cells. Cells were treated with anti-DNP antibody, PBMC, and either ARM-P8 or TNP treatment as indicated. No cytotoxicity was observed for unlabeled DU145 cells at any concentration of ARM-P8, but TNP-labeled cells were susceptible to antibody-mediated lysis by PBMC. (C) Competition ADCC experiments. Here 2-PMPA was added at increasing concentrations to LNCaP cells plus ARM-P8, anti-DNP antibody, and PBMC. As shown, 2-PMPA diminishes ARM-P8-mediated ADCC of LNCaP cells. (D) ARM-P8 Cytotoxicity Studies. LNCaP cells were incubated with ARM-P8 and anti-DNP antibody in the absence of PBMC. These data demonstrate that effector cells are required for ARM-P8-mediated cytotoxicity. (E) ADCC dose curves at various Ab concentrations. LNCaP cells were incubated with ARM-P8 and PBMC (PBMC Lot# PCA1599B, 50:1 effector:target ratio) at varying anti-DNP antibody concentrations as indicated. These results indicate a dependence of antibody concentration on the concentration of ARM-P at which auto-inhibition sets in. Thus, the maximal degree of cell killing is roughly proportional to antibody concentration, and the ARM-P concentration at which cell-killing curves begin to decline increases with increasing antibody concentrations.

Extended Reference List

(12) Slusher, B. S.; Vornov, J.; Thomas, A. G.; Hurn, P. D.; Harkuni, I.; Bhardwaj A.l Traystman, R. J.; Robinson, M. B.; Britton, P.; Lu, X. C. M.; Tortella, F. C.; Wozniak, S. K. M.; Yudkoff, M.; Potier, B. M.; Jackson, P. F. *Nat. Med.* **1999**, *5*, 1396-1402.

^{1}H NMR Spectra for **s-2a** ($CDCl_3$)



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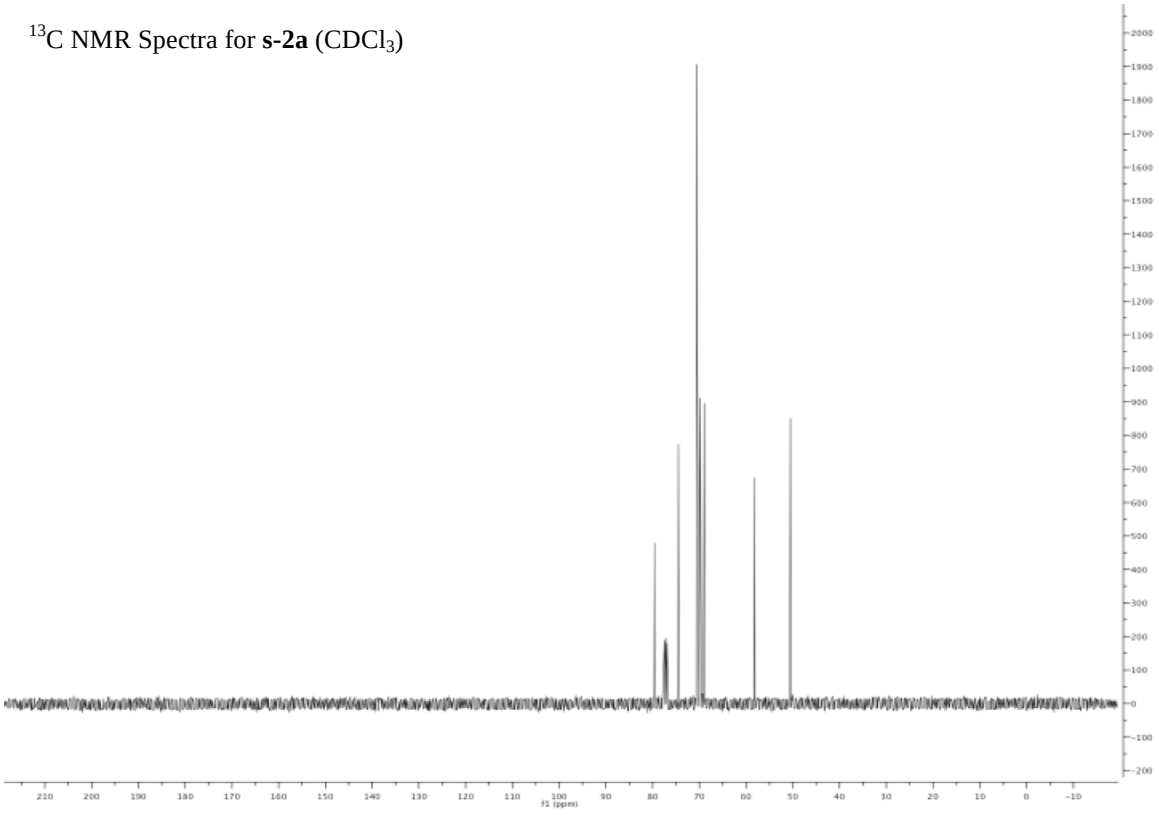

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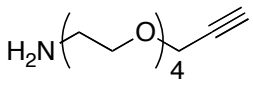

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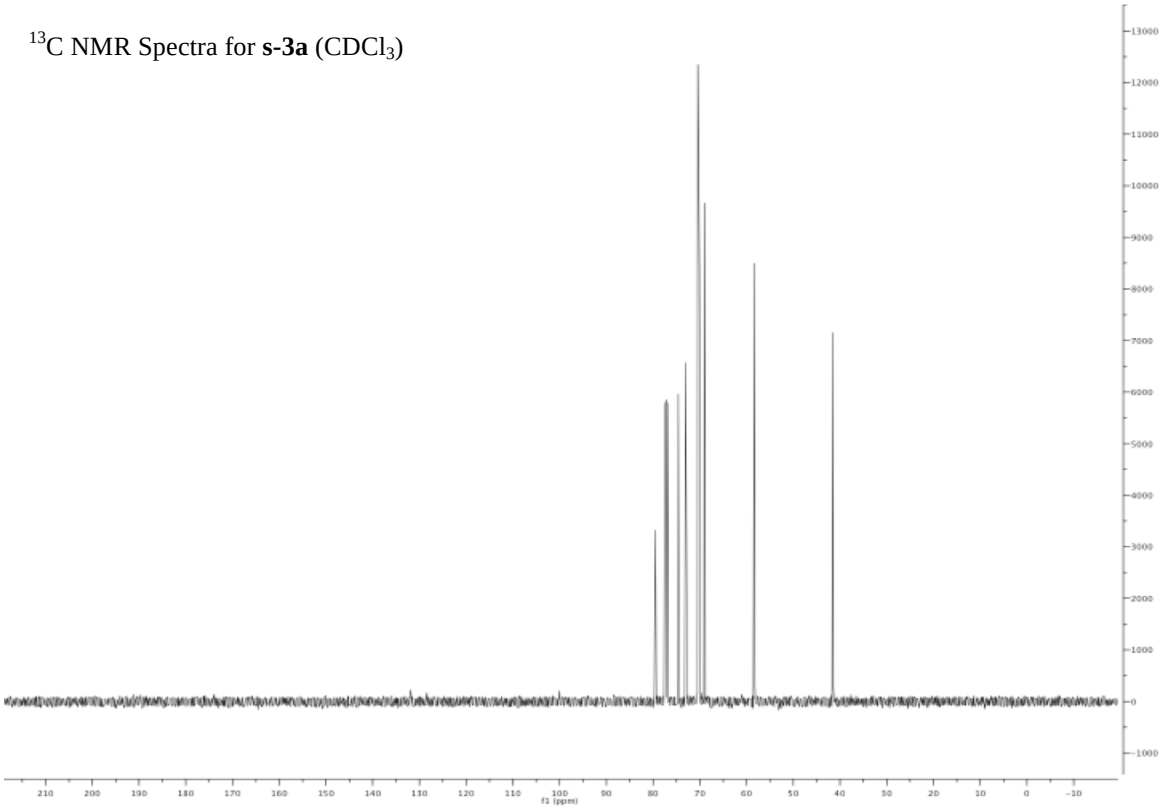

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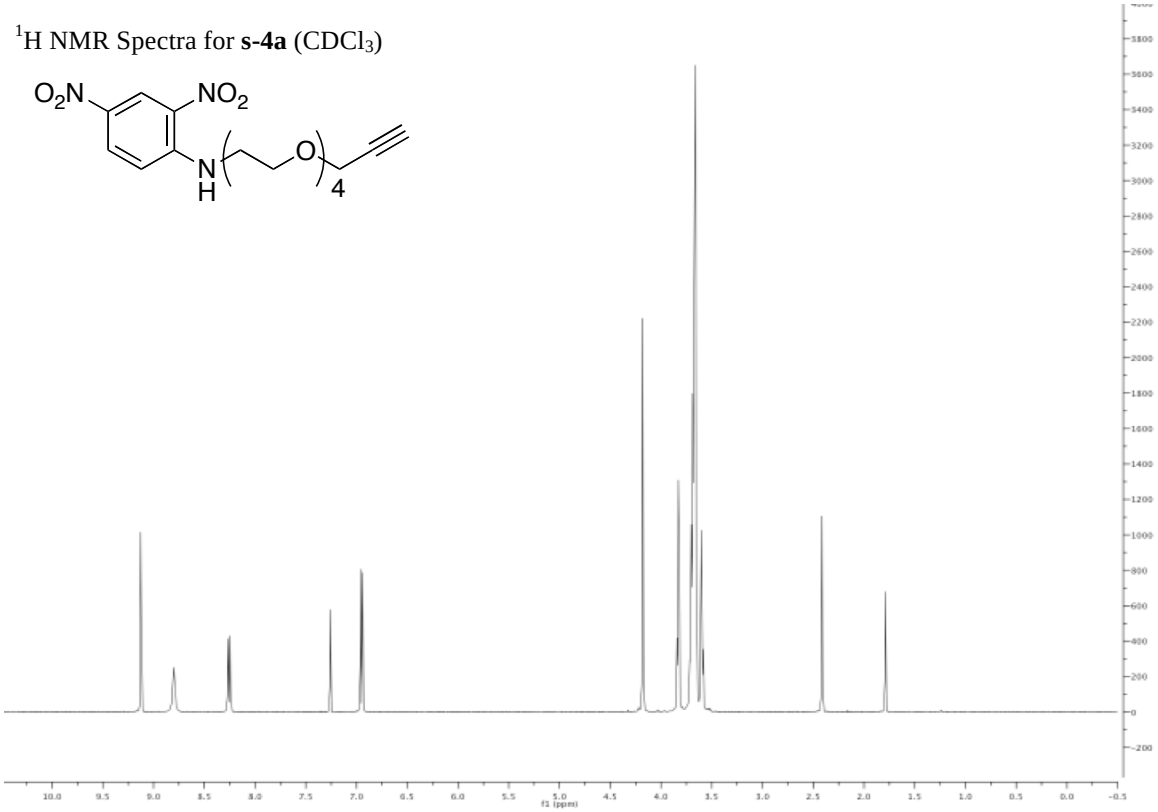

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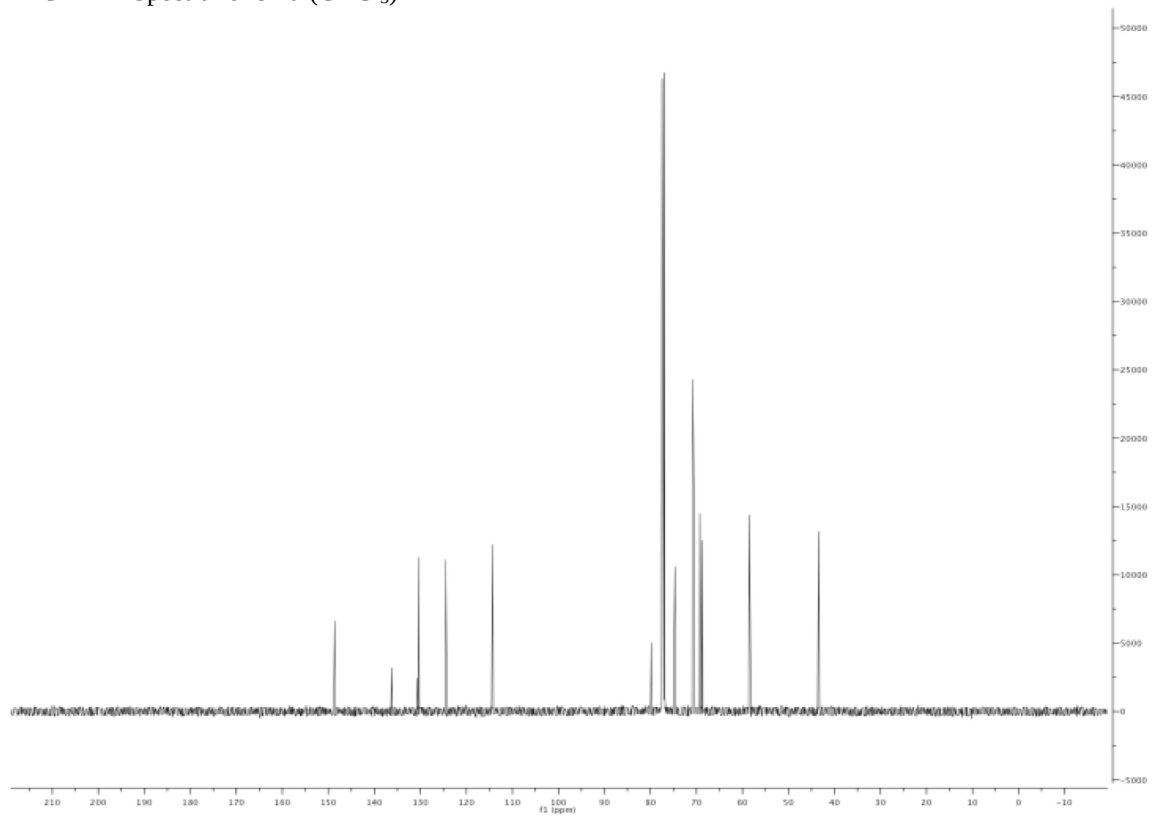

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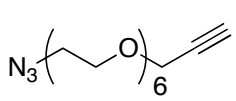

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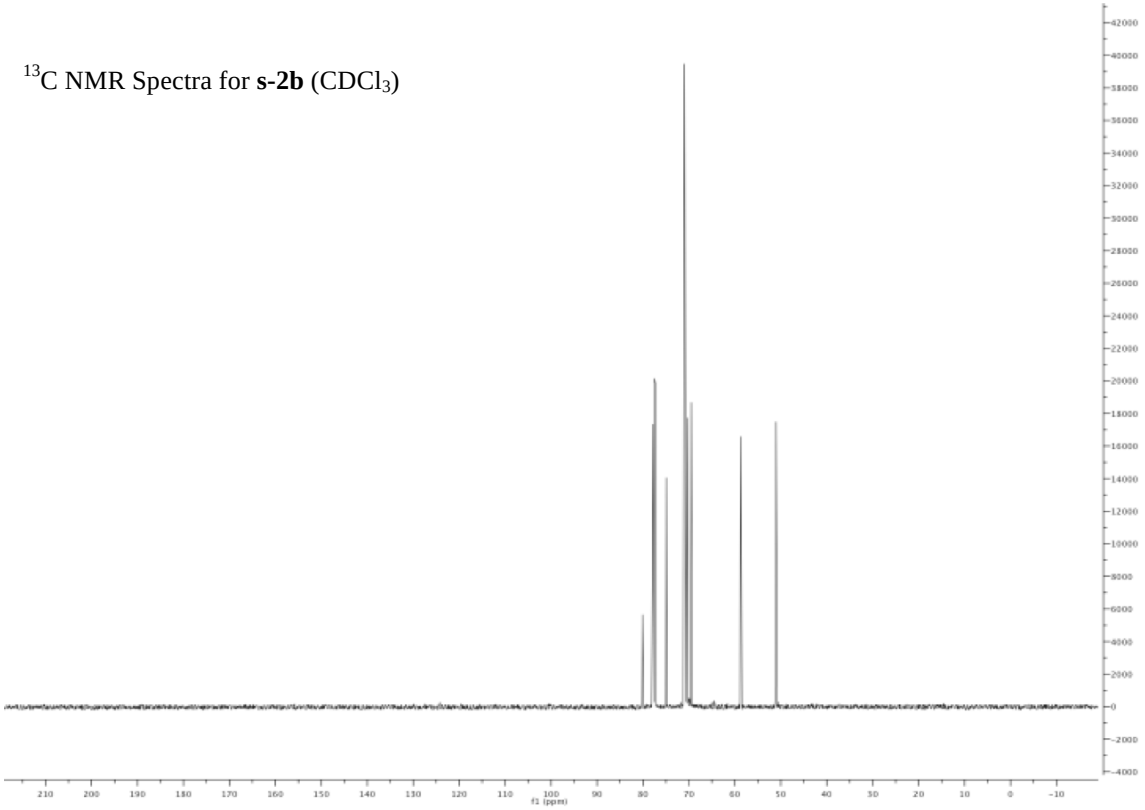

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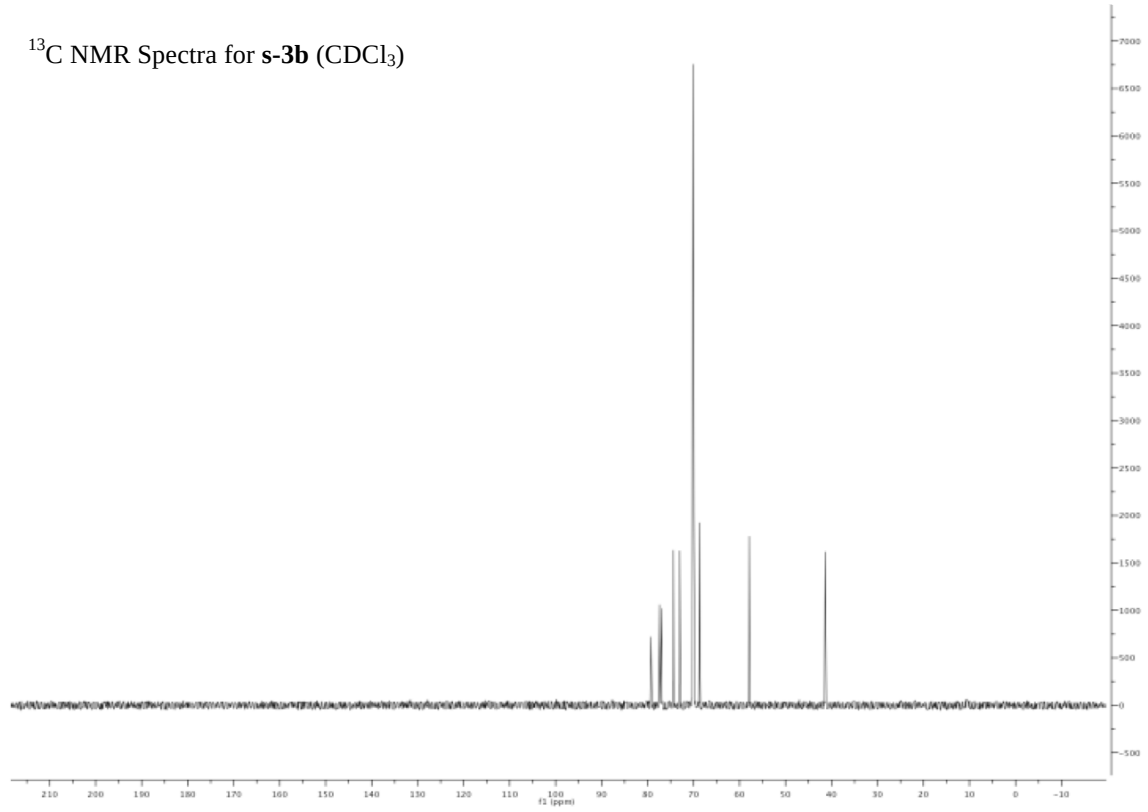

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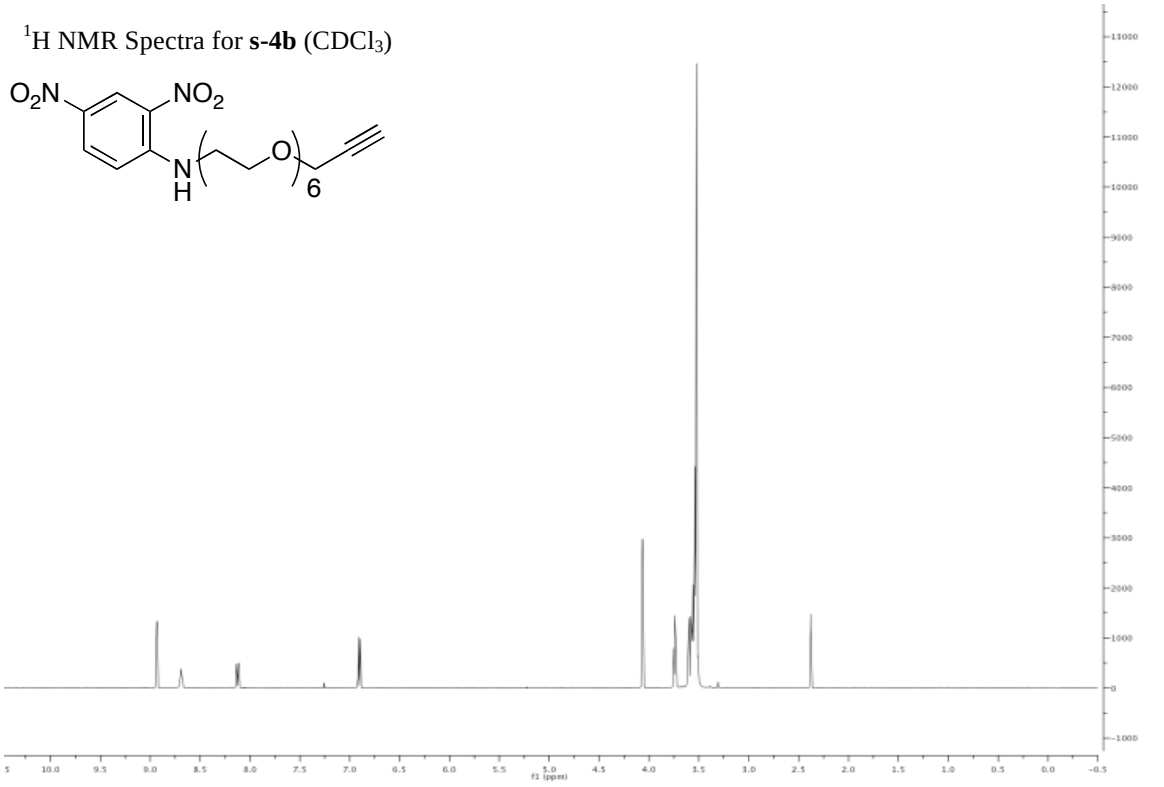

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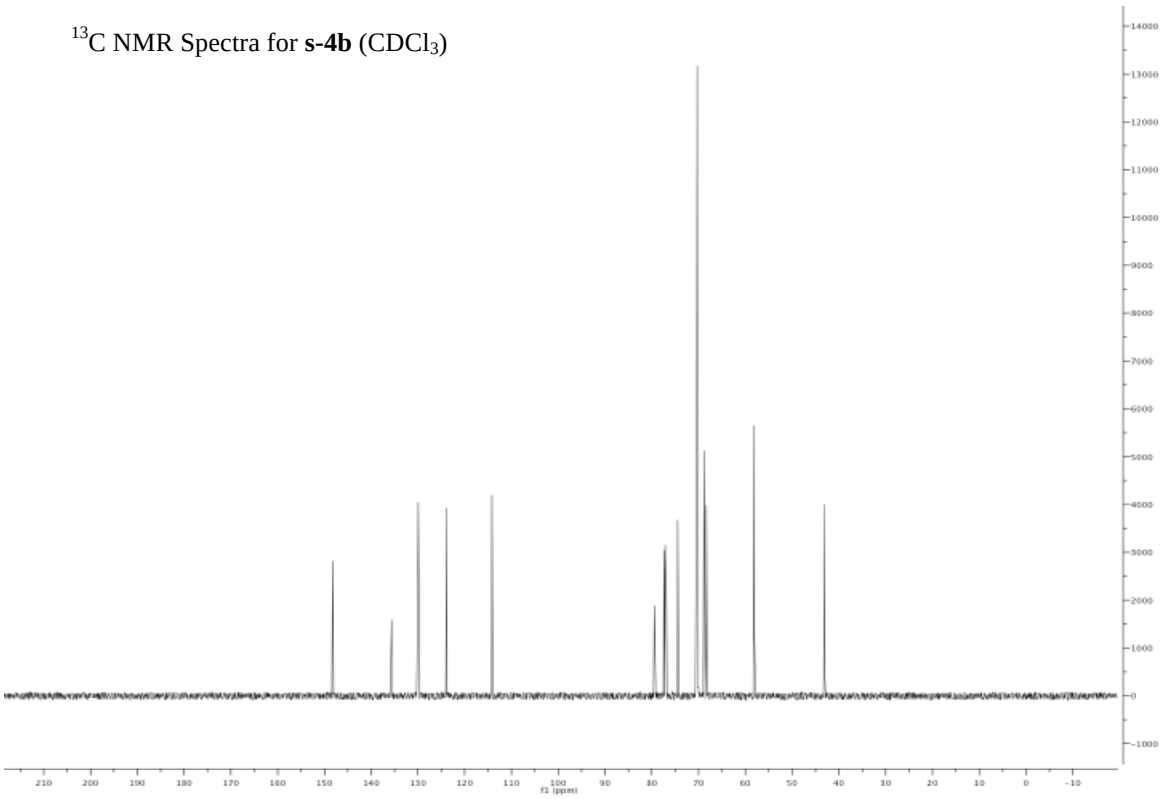

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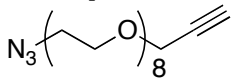

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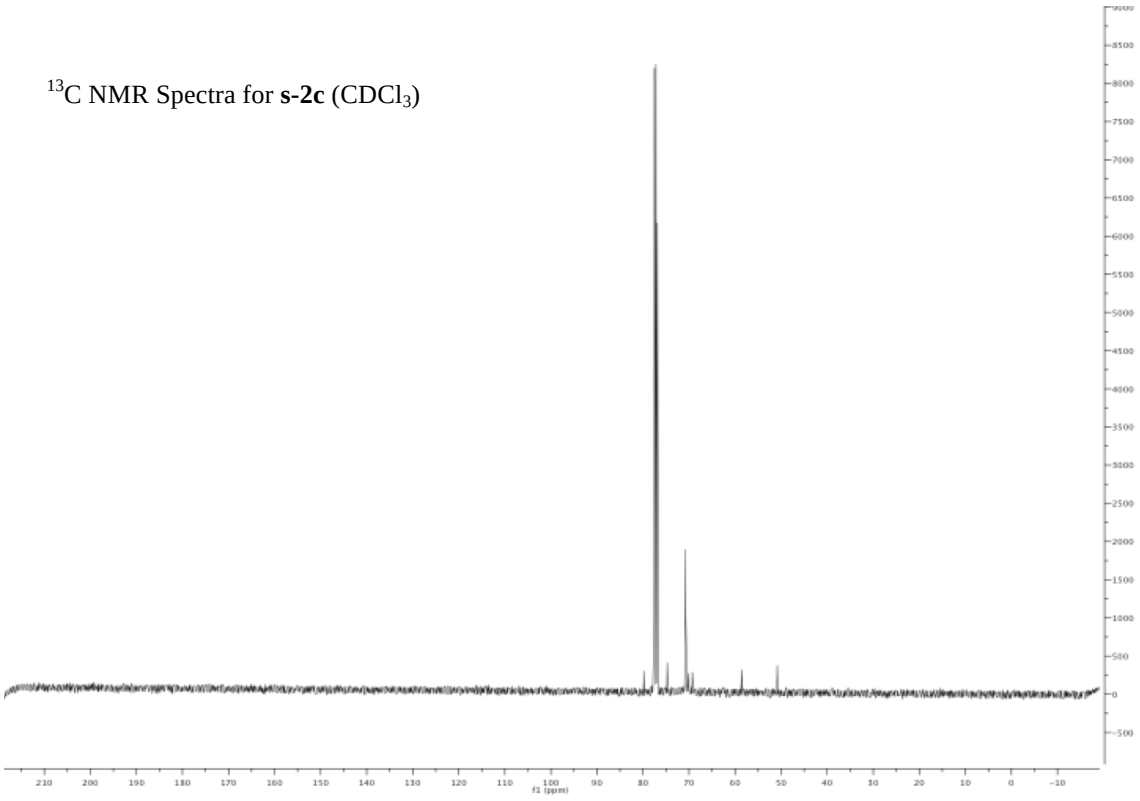

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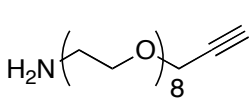

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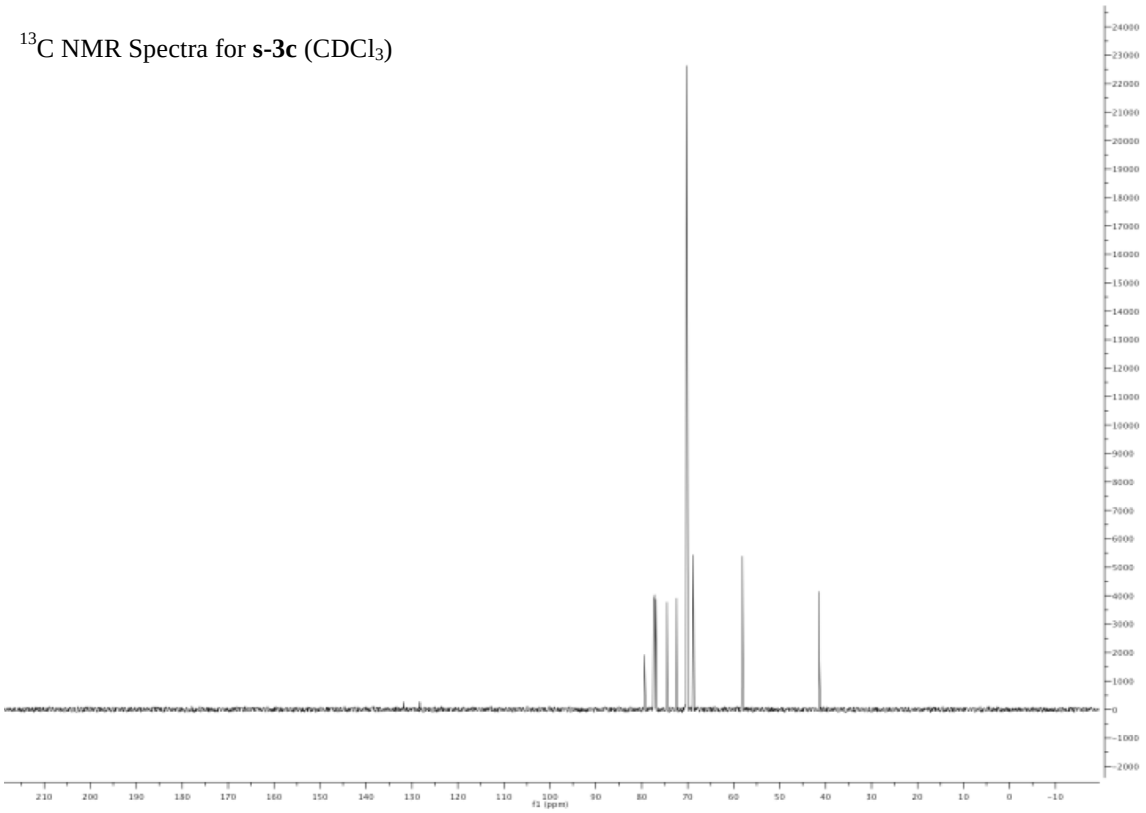

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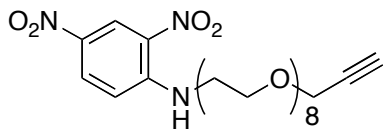

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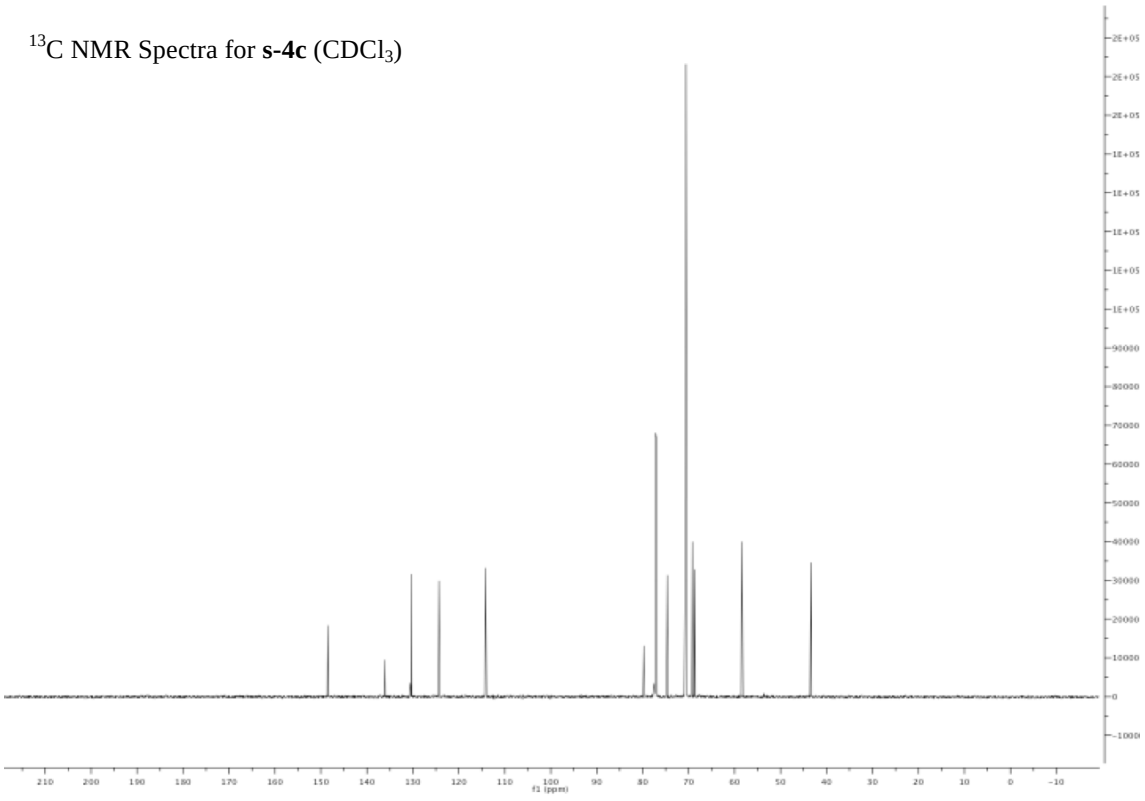

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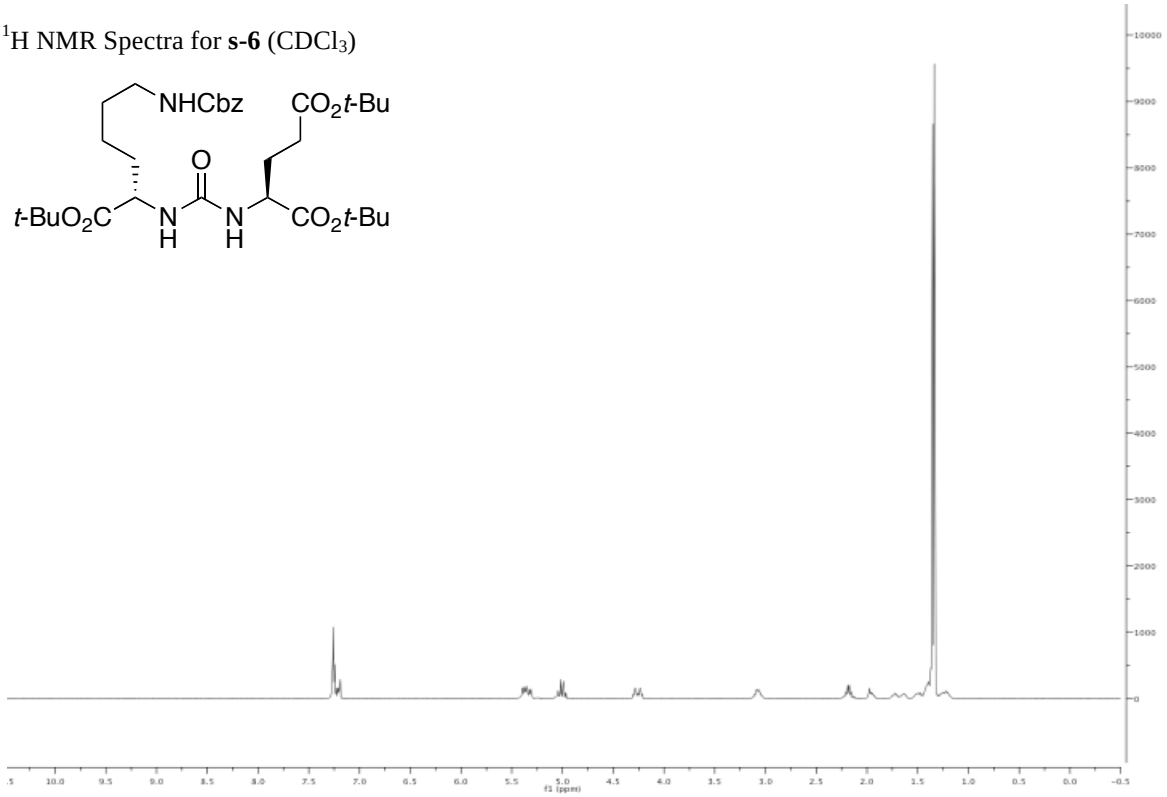

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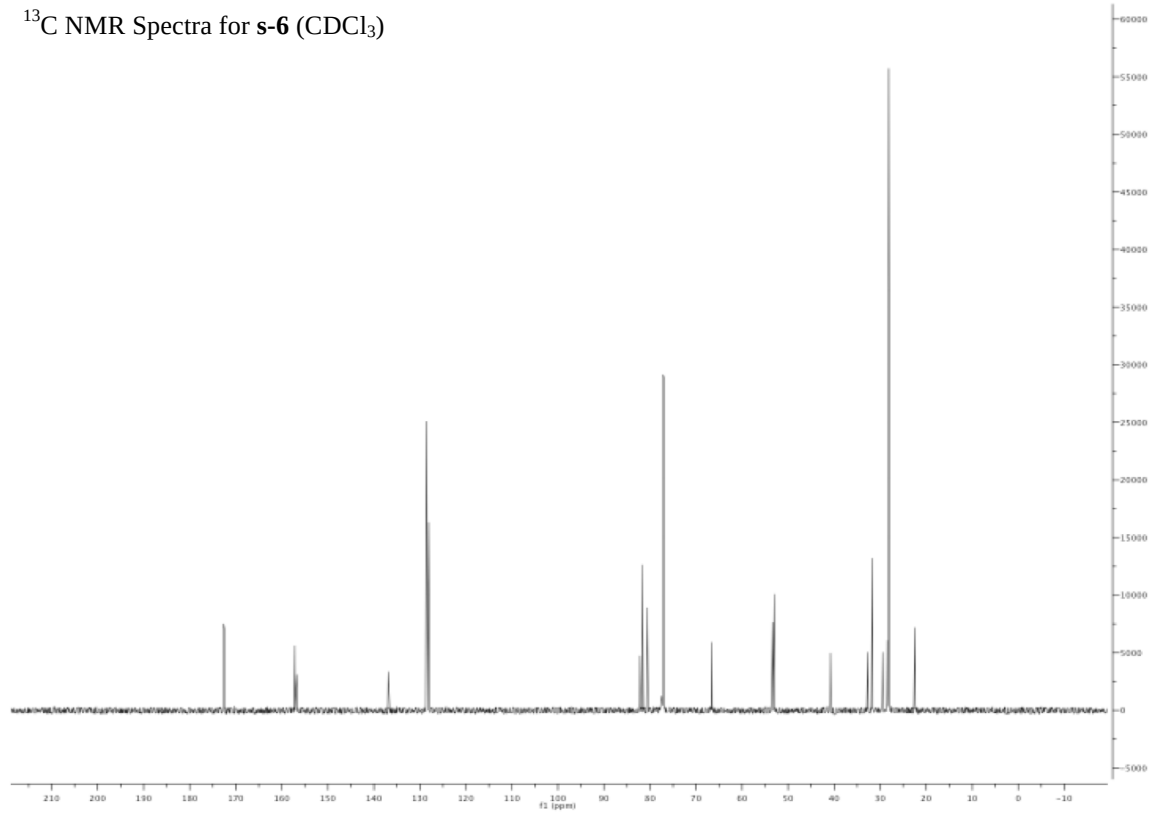

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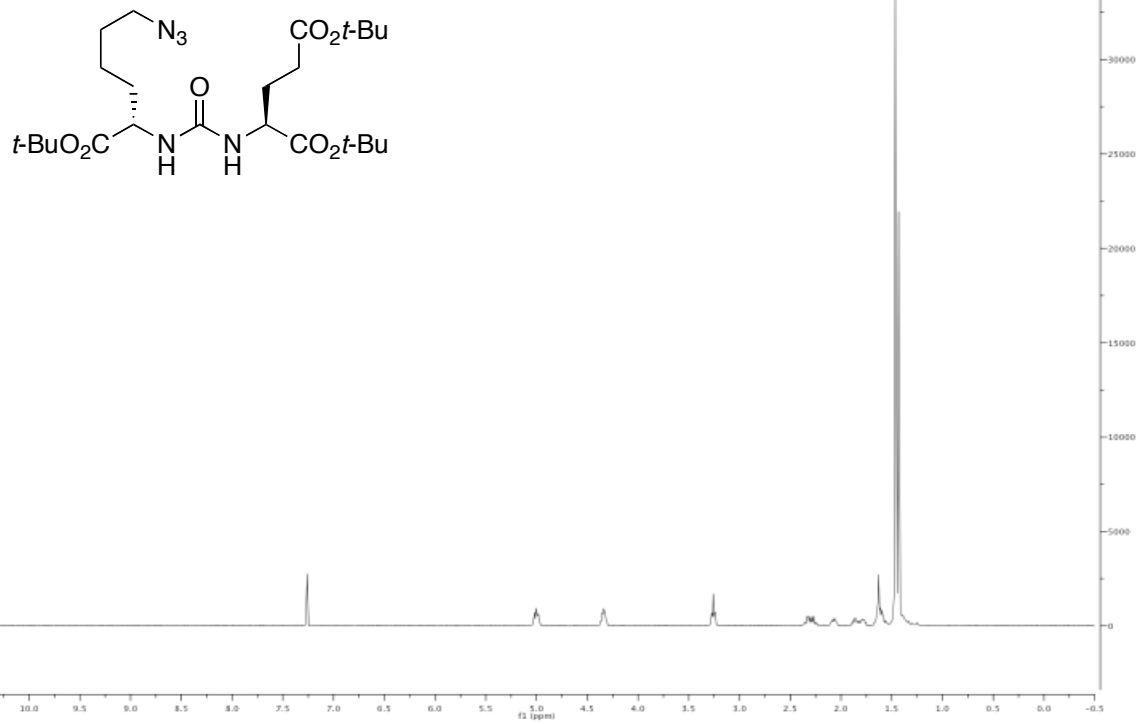

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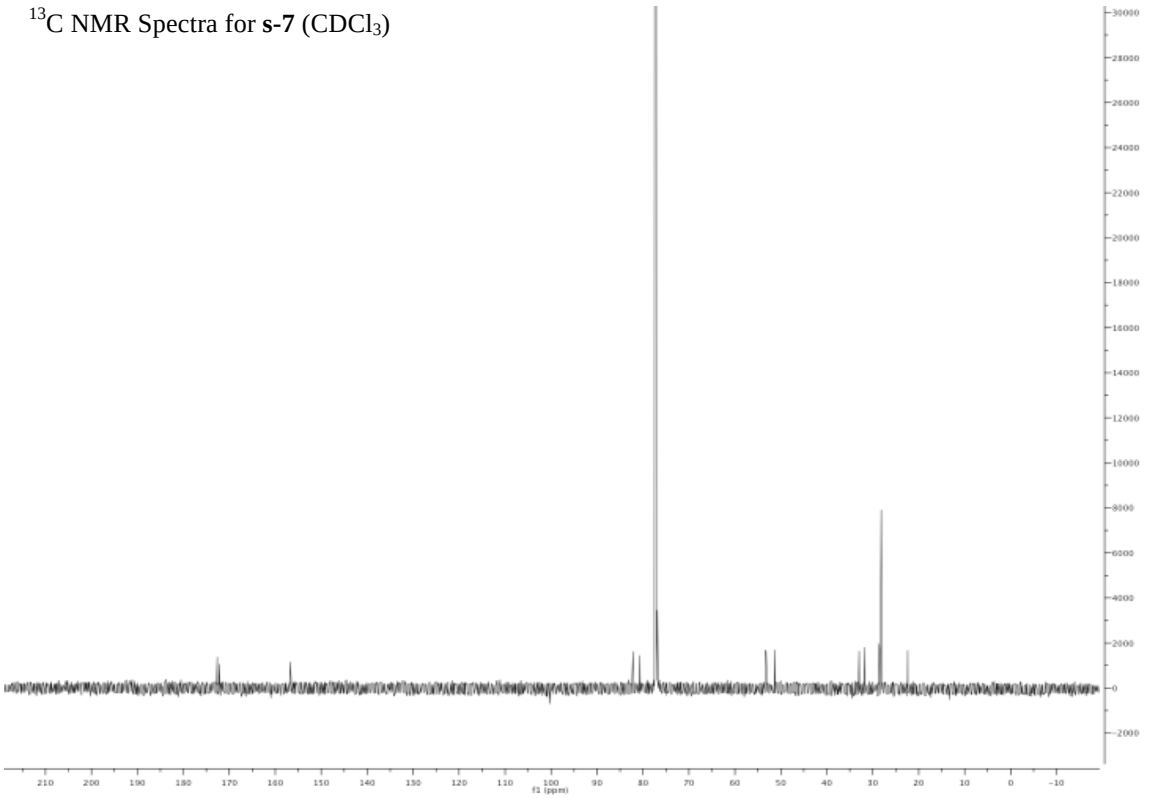

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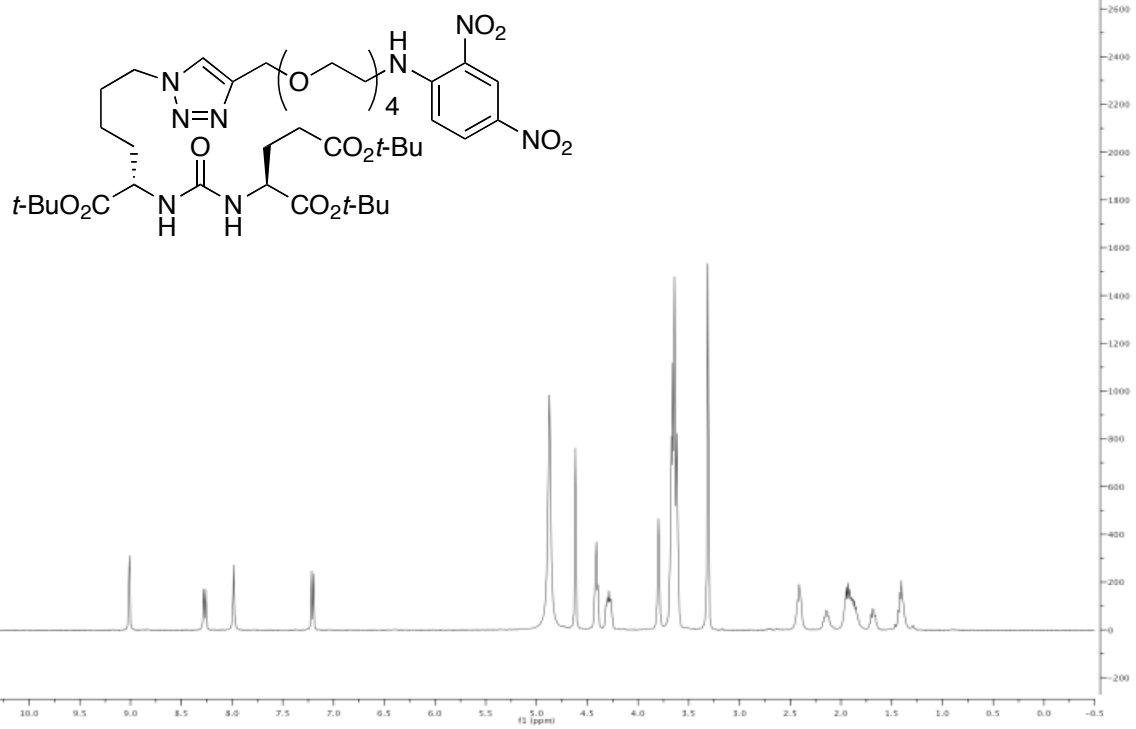

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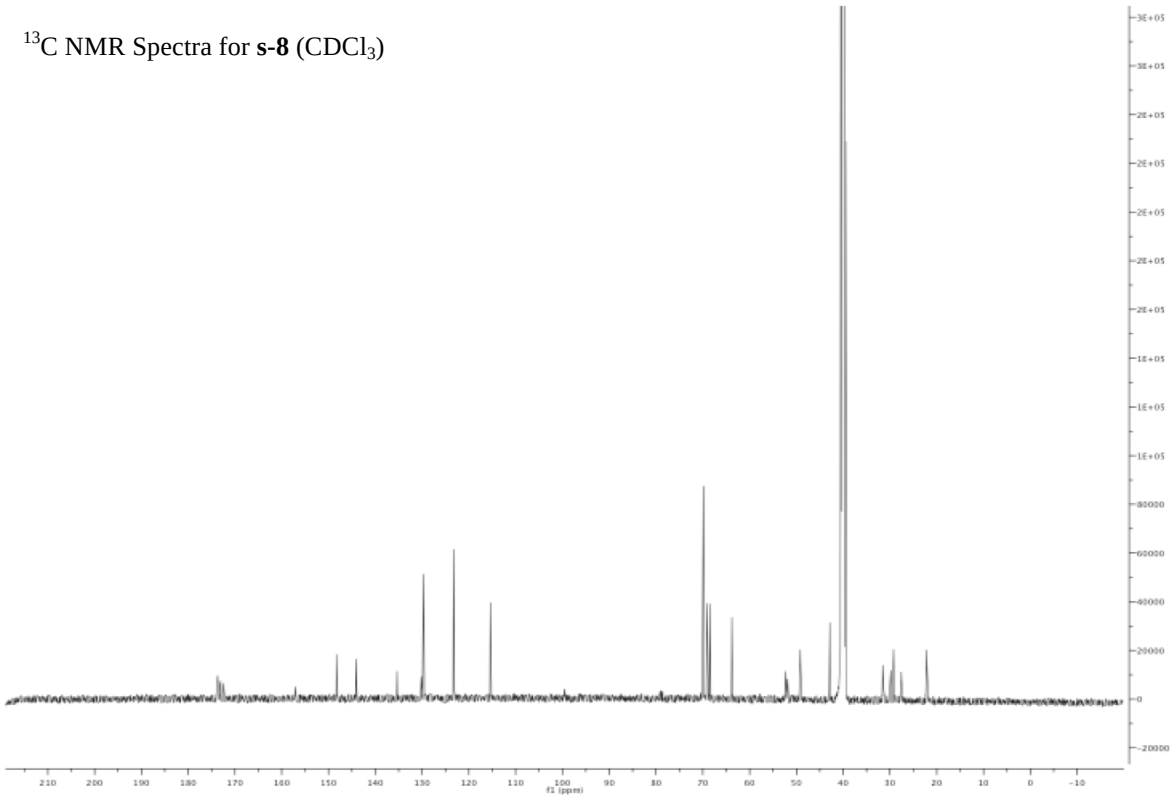

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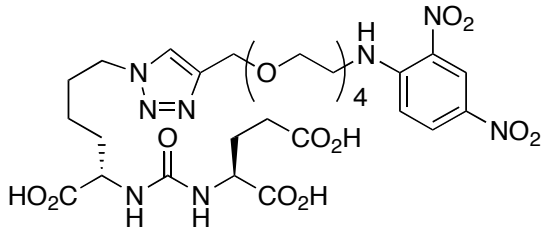
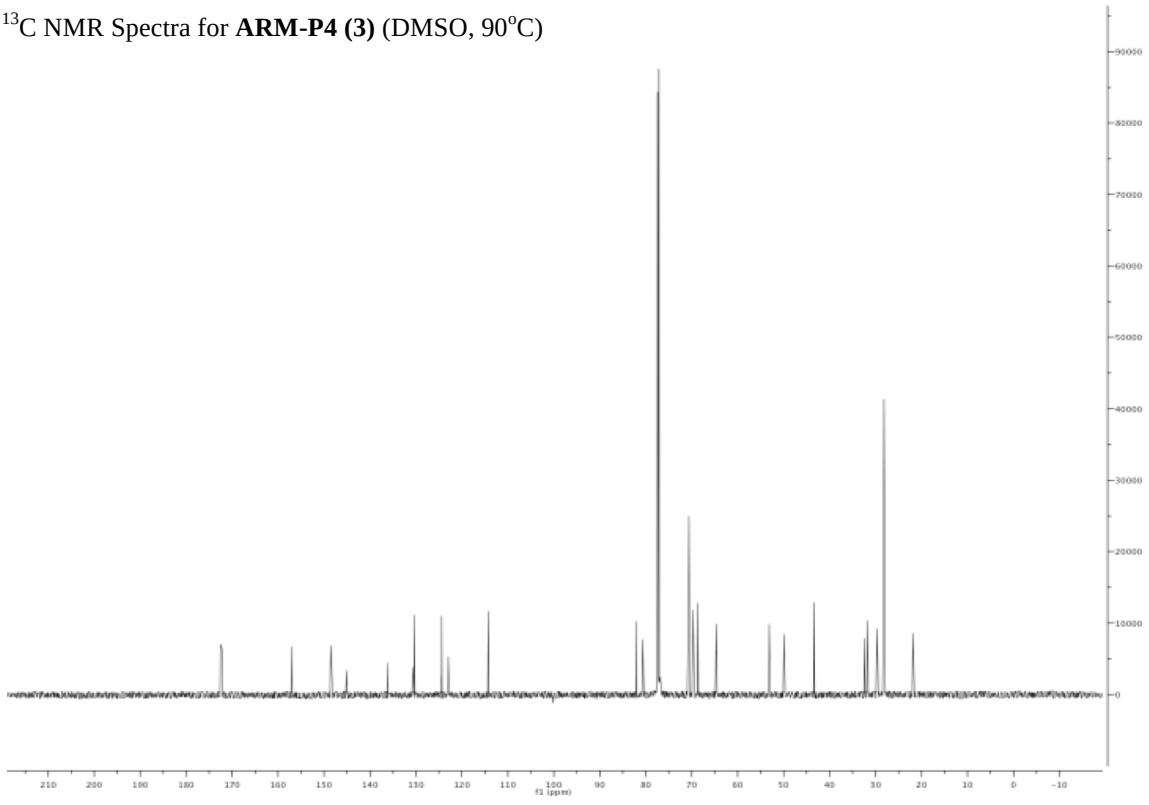
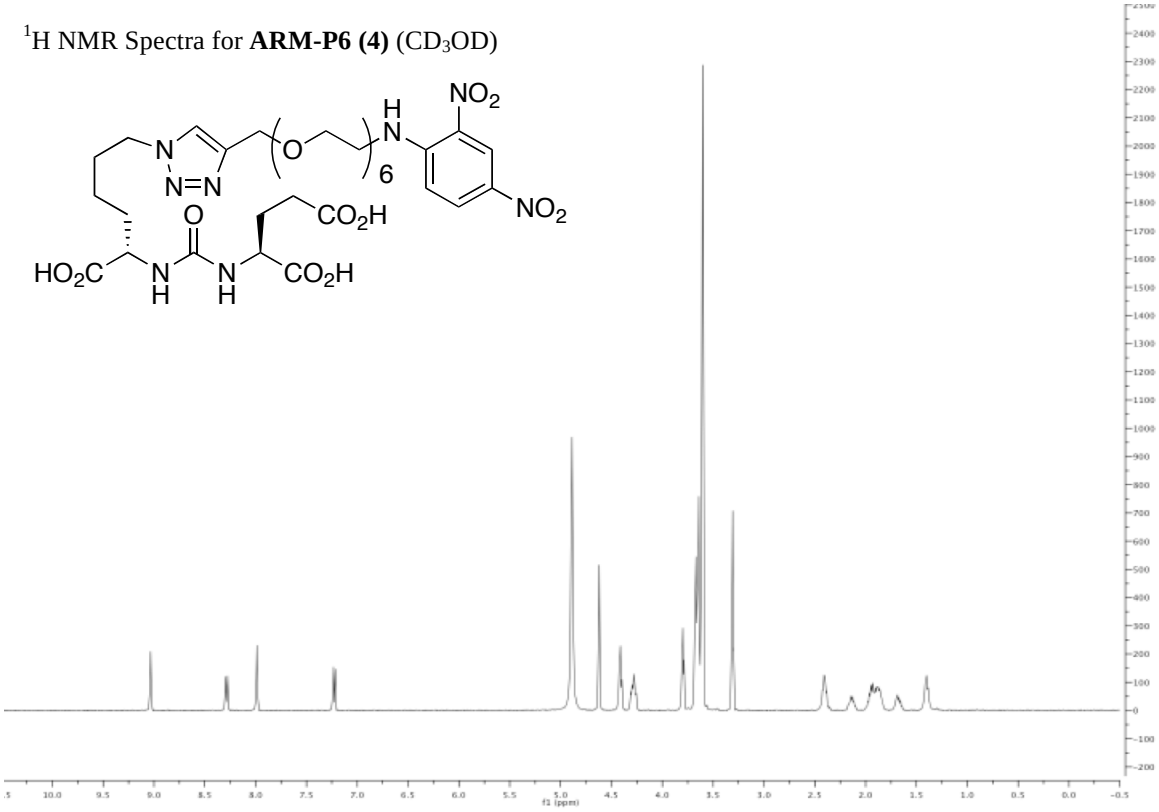


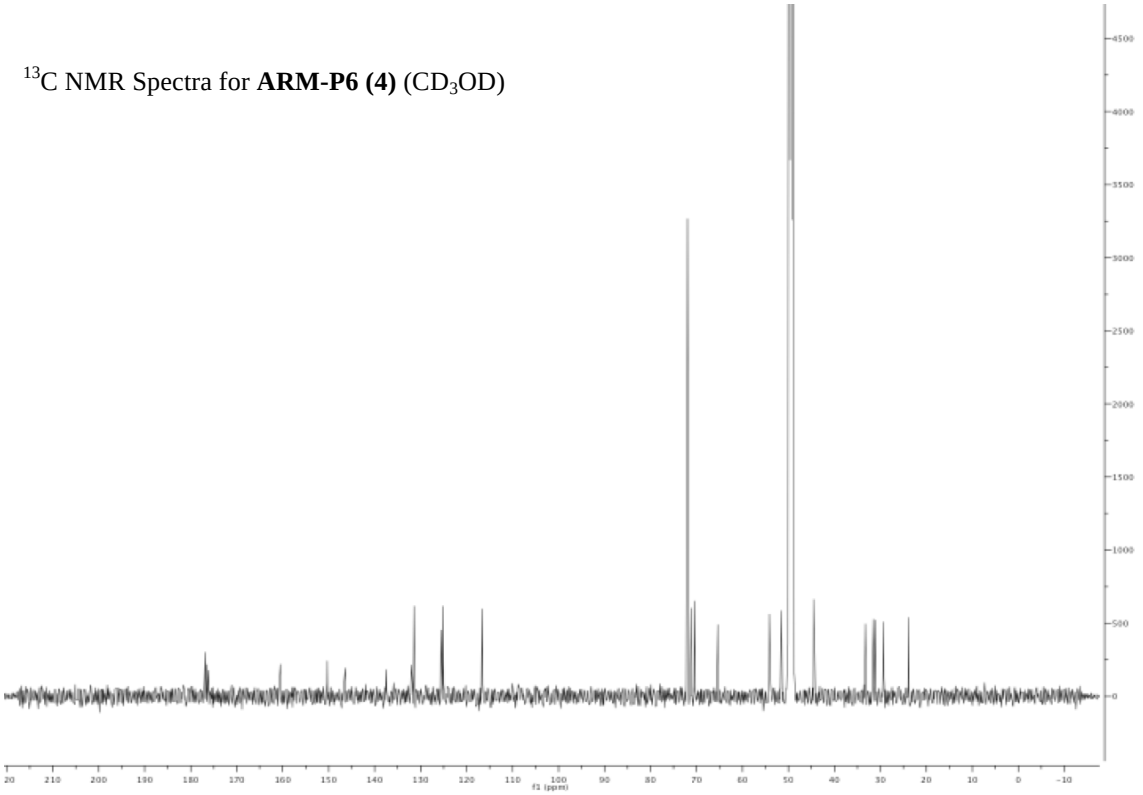
^{13}C NMR Spectra for **ARM-P4 (3)** (DMSO, 90°C)



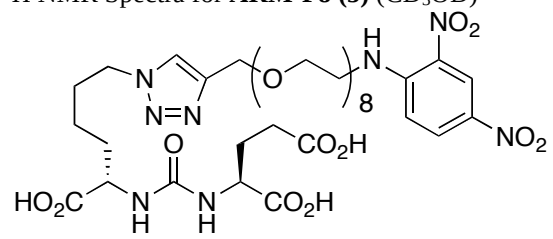
^{1}H NMR Spectra for **ARM-P6 (4)** (CD_3OD)



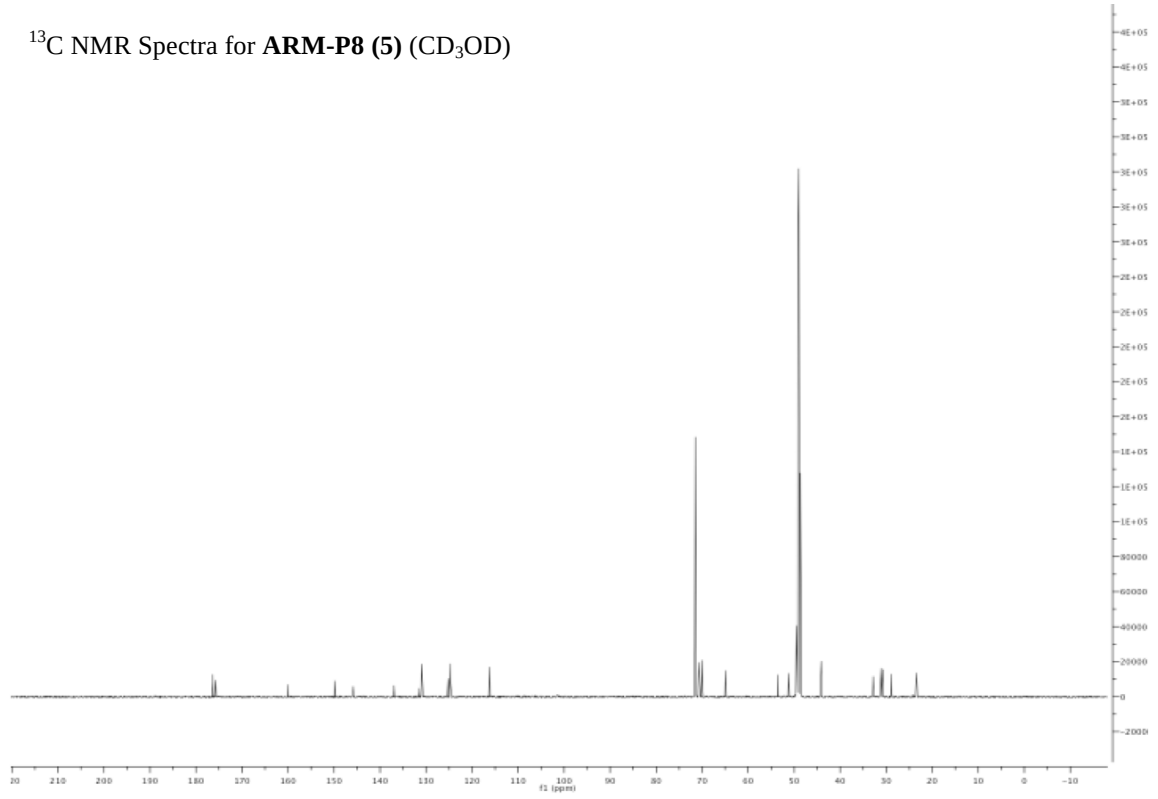
^{13}C NMR Spectra for **ARM-P6 (4)** (CD_3OD)



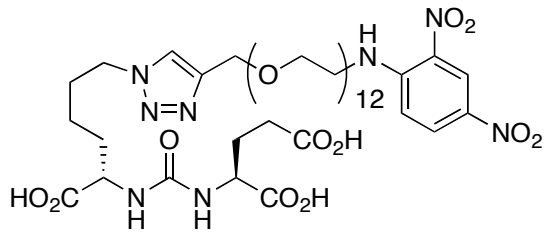
^{1}H NMR Spectra for **ARM-P8 (5)** (CD_3OD)



^{13}C NMR Spectra for **ARM-P8 (5)** (CD_3OD)



^{1}H NMR Spectra for **ARM-P12 (6)** (CD_3OD)



^{13}C NMR Spectra for **ARM-P12 (6)** (d-DMSO)