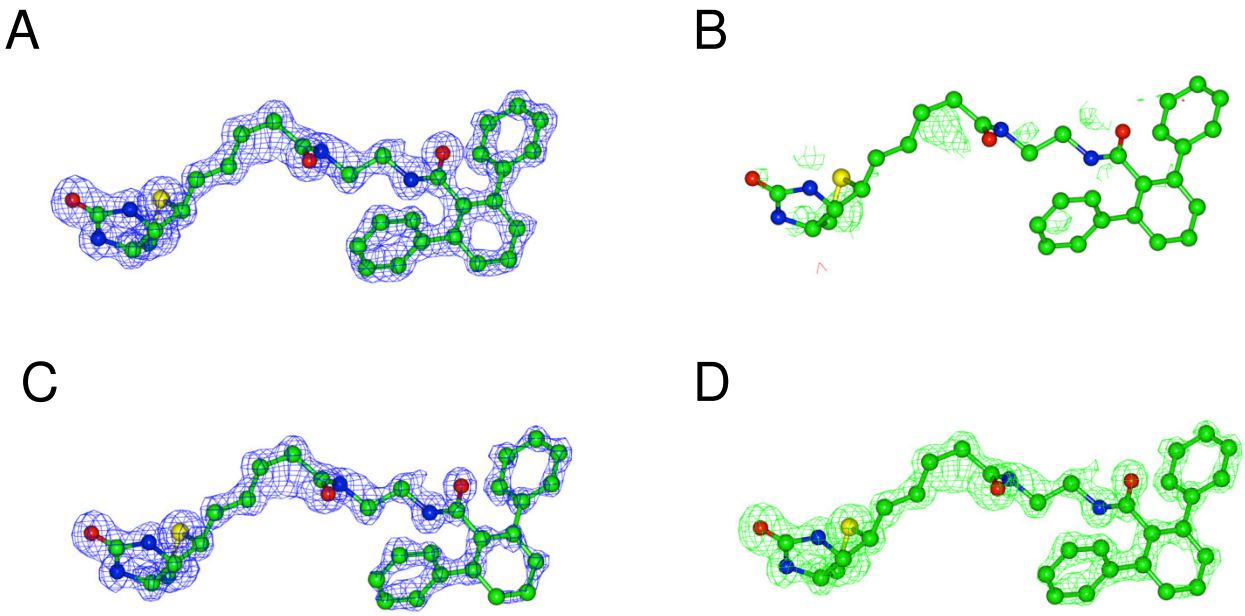
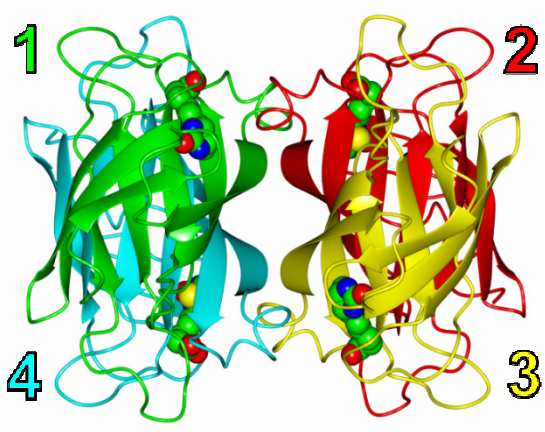


Figure S1. Electron density around the LH1 ligand in streptavidin (chain B of the crystal structure). (A) The 2mFo-DFc map contoured at 1 σ. (B) The mFo-DFc map with negative (red) and positive (green) density contoured at 3 σ. (C) The 2mFo-DFc simulated annealing omit map contoured at 1 σ. (D) The mFo-DFc simulated annealing omit map with negative (red) and positive (green) density contoured at 3 σ.

A

Wild-type Streptavidin (tetravalent)



B

Trans-divalent (1,3)

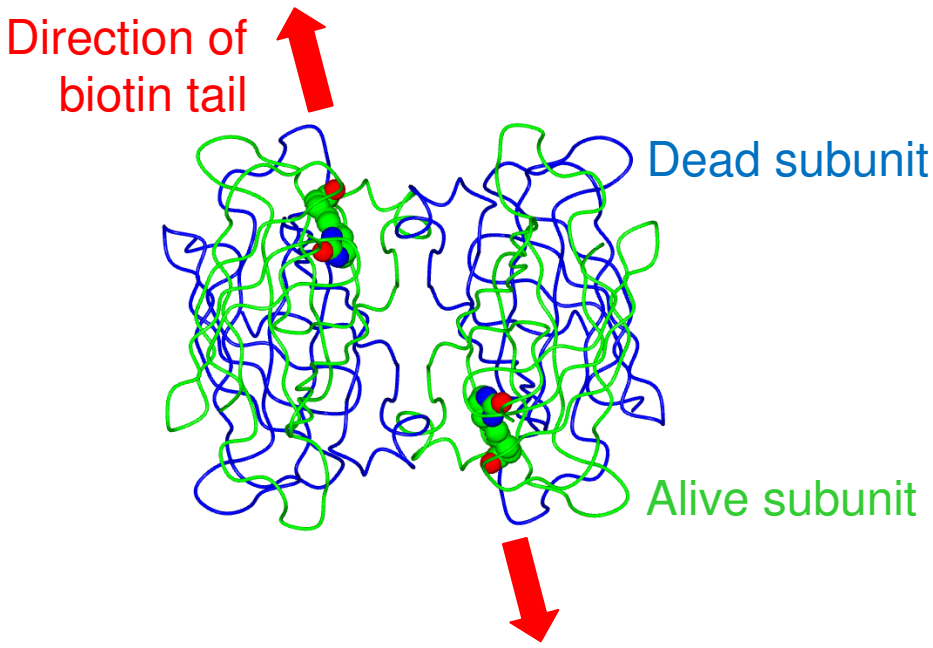


Figure S2. Organization of streptavidin tetramer. (A) Numbering of streptavidin subunits, with each subunit in a different color in cartoon format and with biotin shown in spacefill. (B) Organization of biotin in 1,3 trans-divalent streptavidin. Alive subunits at the 1 and 3 positions are in green ribbon format, Dead subunits at the 2 and 4 positions are in blue ribbon format, and biotin is in spacefill. Structures based on PDB 3RY2.

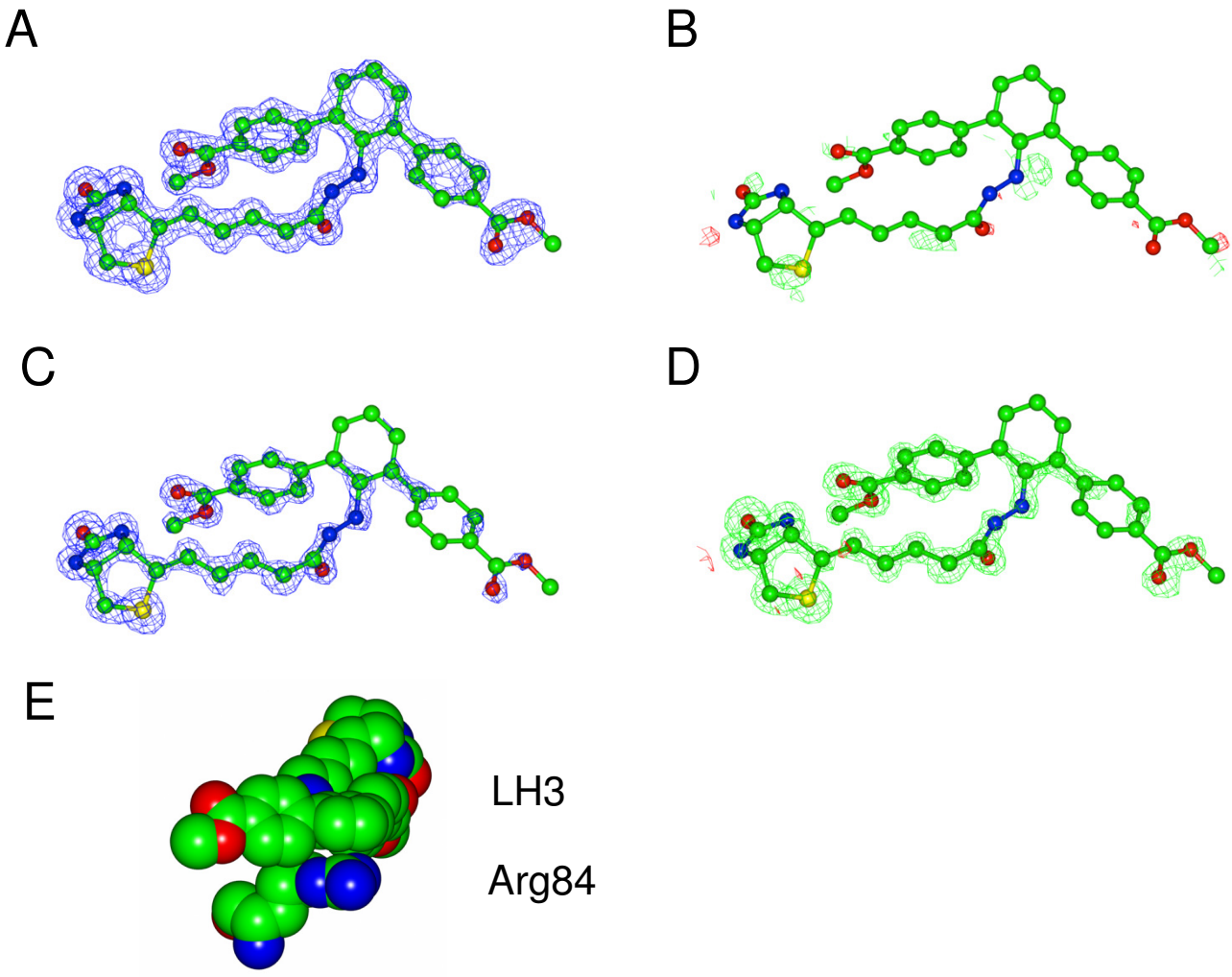


Figure S3. Electron density and contacts around the LH3 ligand in streptavidin (Chain B of the crystal structure). (**A**) The 2mFo-DFc map contoured at 1 σ. (**B**) The mFo-DFc map with negative (red) and positive (green) density contoured at 3 σ. (**C**) The 2mFo-DFc simulated annealing omit map contoured at 1 σ. (**D**) The mFo-DFc simulated annealing omit map with negative (red) and positive (green) density contoured at 3 σ. (**E**) Putative cation-π interaction of Arg84 of streptavidin with LH3, shown as the van der Waals surface.

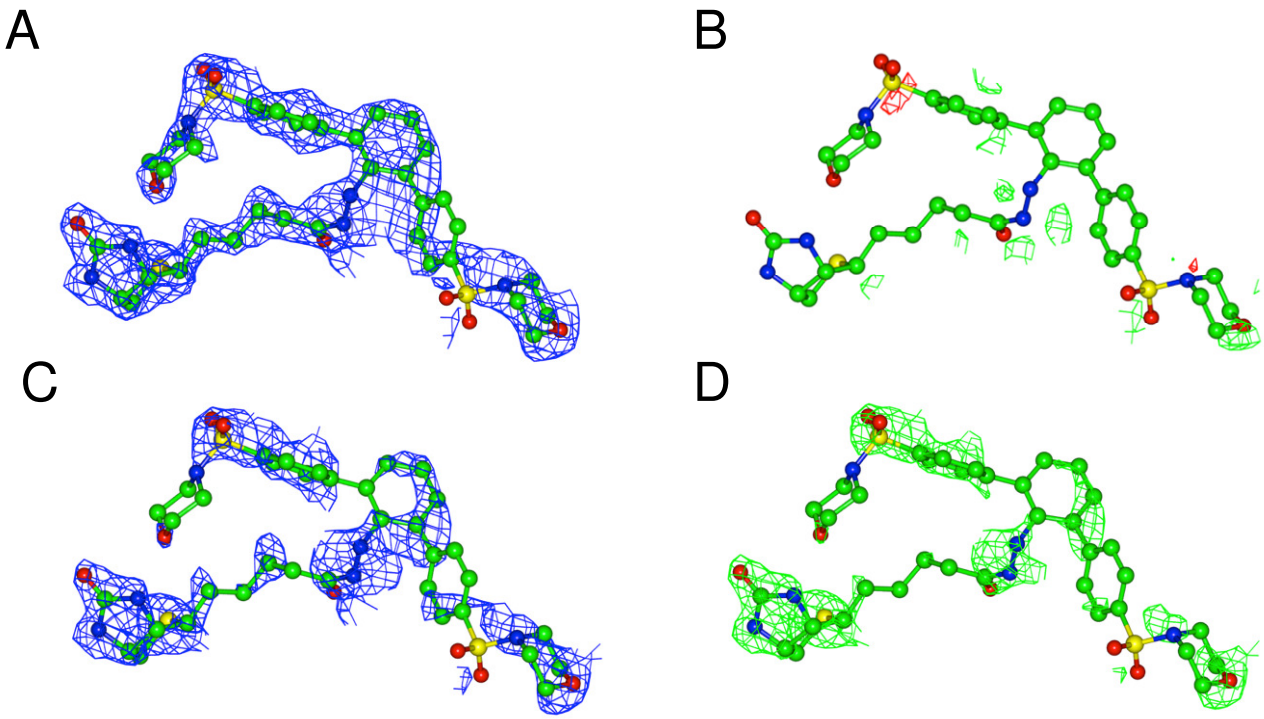


Figure S4. Electron density around the LH4 ligand in trans-divalent streptavidin (Chain C of the crystal structure). (A) The 2mFo-DFc map contoured at 1 σ. (B) The mFo-DFc map with negative (red) and positive (green) density contoured at 3 σ. (C) The 2mFo-DFc simulated annealing omit map contoured at 1 σ. (D) The mFo-DFc simulated annealing omit map with negative (red) and positive (green) density contoured at 3 σ.

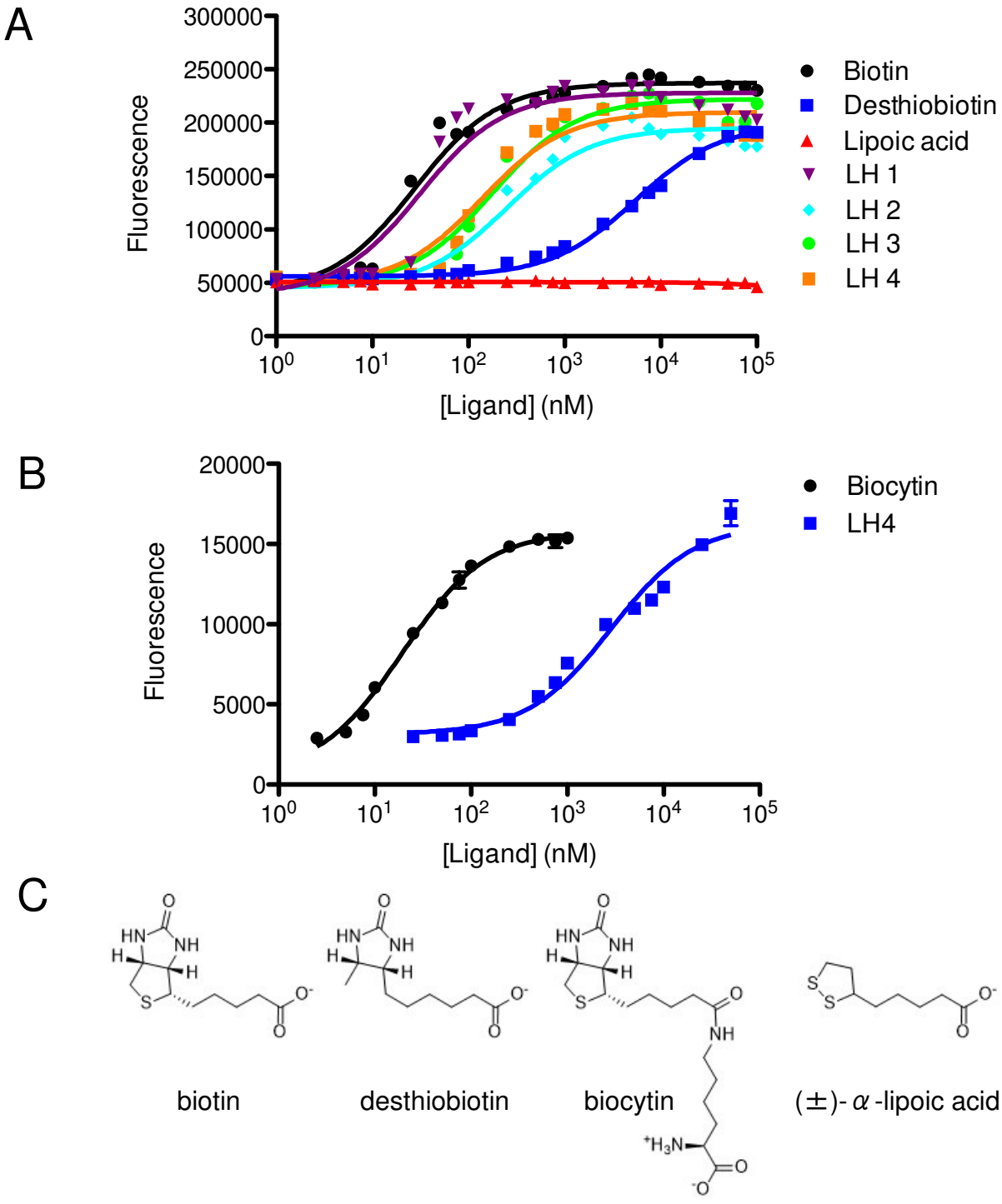


Figure S5. Streptavidin-binding properties of ligands. (A) 10 nM biotin-4-fluorescein was incubated with 50 nM monovalent streptavidin and the indicated concentration of each ligand for 48 h at 37 °C and fluorescence was measured. (B) Titration as for (A) except using A86D monovalent streptavidin, showing mean of triplicate ±1 s.d. Some error bars are too small to be visible. (C) Chemical structure of biotin alongside desthiobiotin, biocytin and (±)-α-lipoic acid.

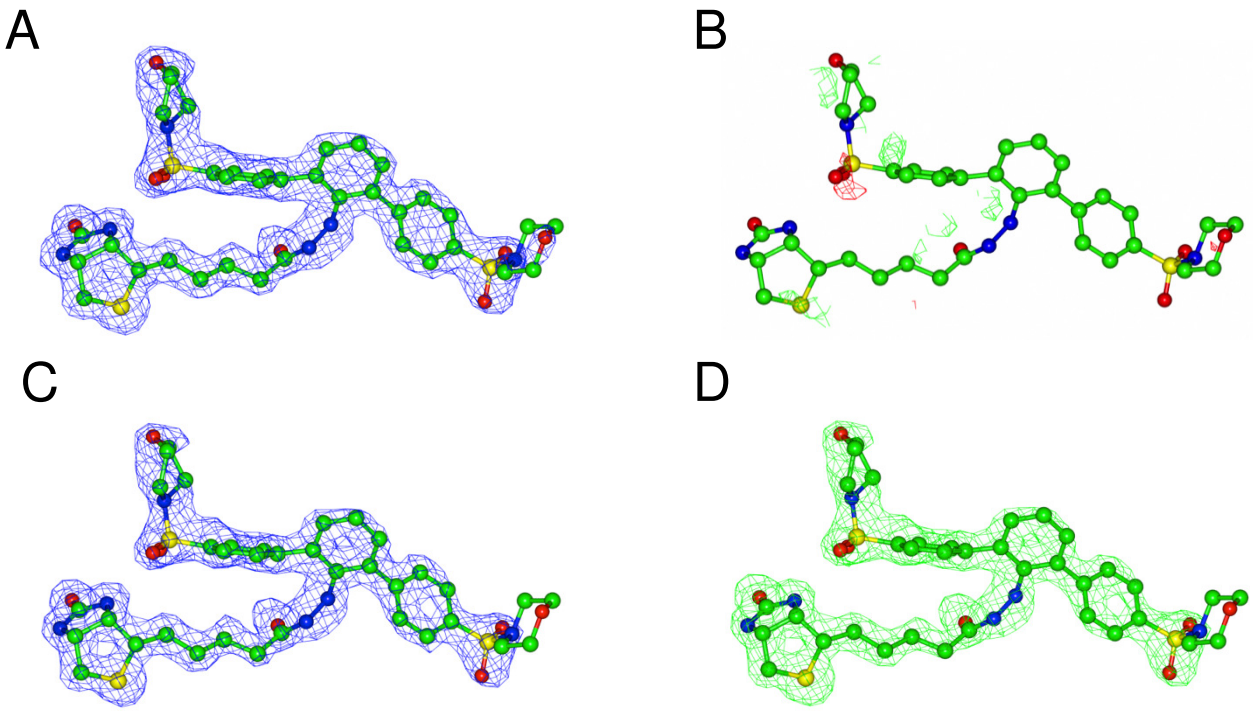


Figure S6. Electron density around the LH4 ligand in streptavidin A86D (Chain D of the crystal structure). (A) The 2mFo-DFc map contoured at 1 σ. (B) The mFo-DFc map with negative (red) and positive (green) density contoured at 3 σ. (C) The 2mFo-DFc simulated annealing omit map contoured at 1 σ. (D) The mFo-DFc simulated annealing omit map with negative (red) and positive (green) density contoured at 3 σ.

SUPPLEMENTARY DATA

Love-Hate ligands for high resolution analysis of strain in ultra-stable protein:small molecule interaction

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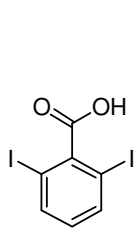
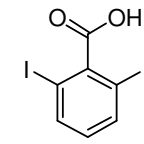
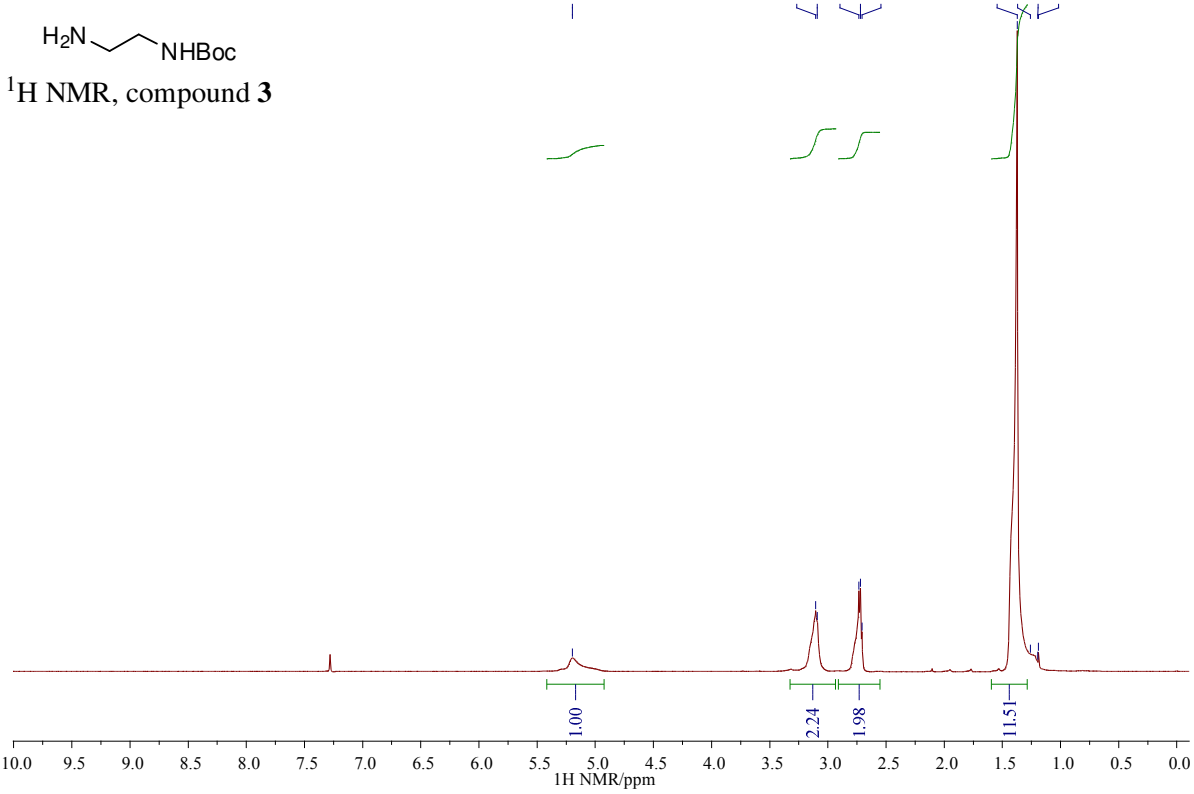
^{1}H and ^{13}C NMR spectra of purified compounds



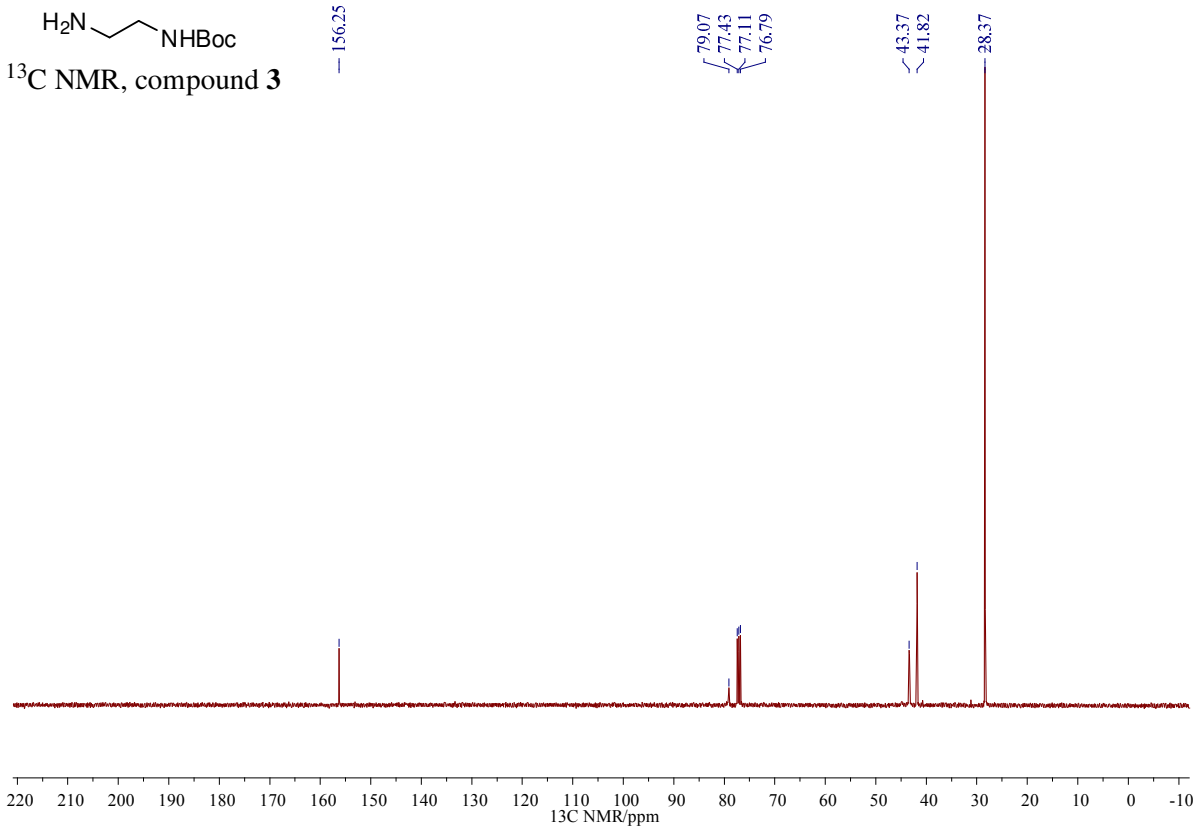
^{1}H NMR, compound **2**



^{13}C NMR, compound **2**



^{1}H NMR, compound **3**



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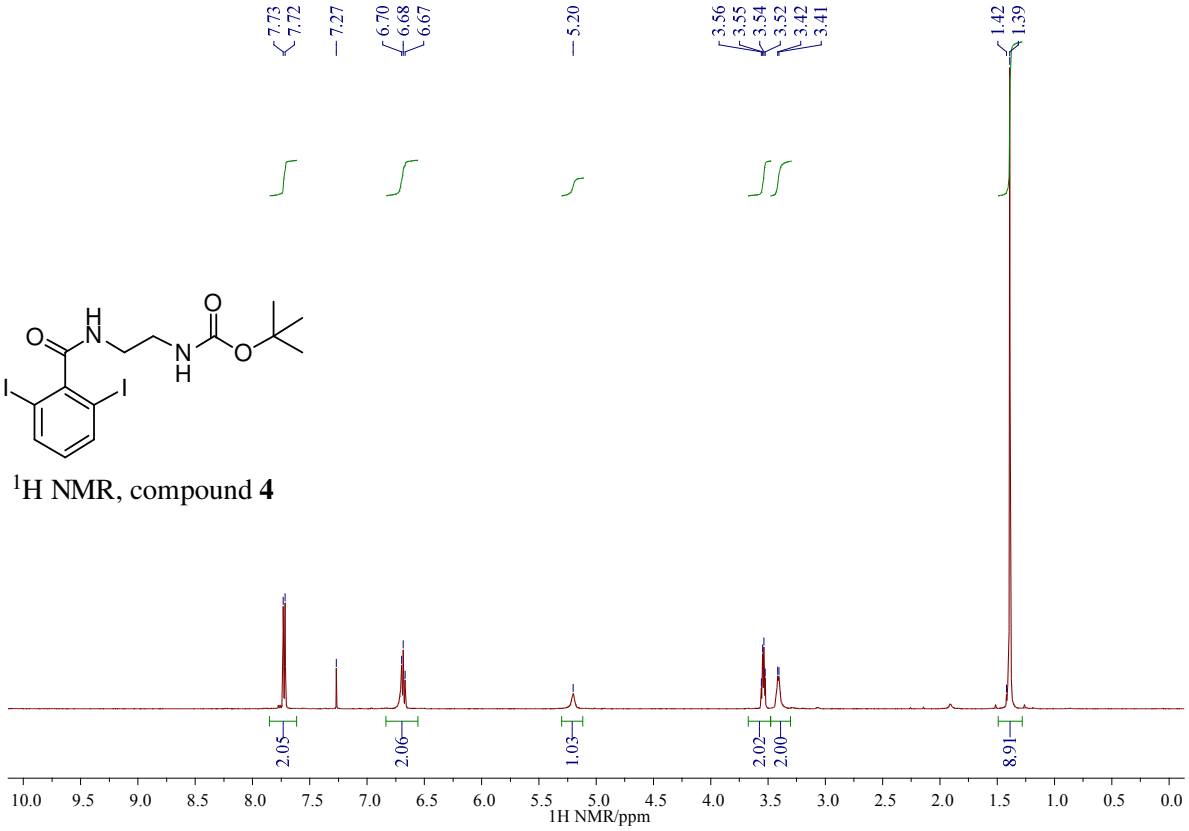

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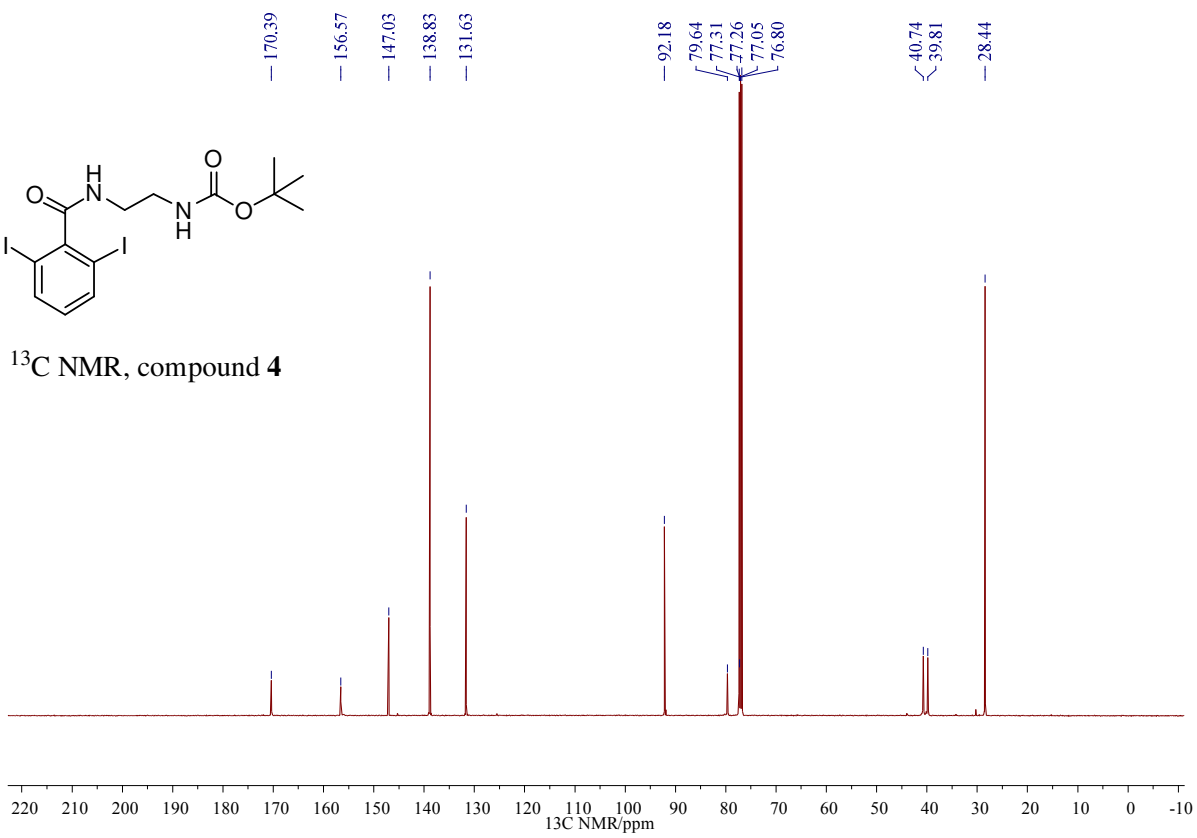
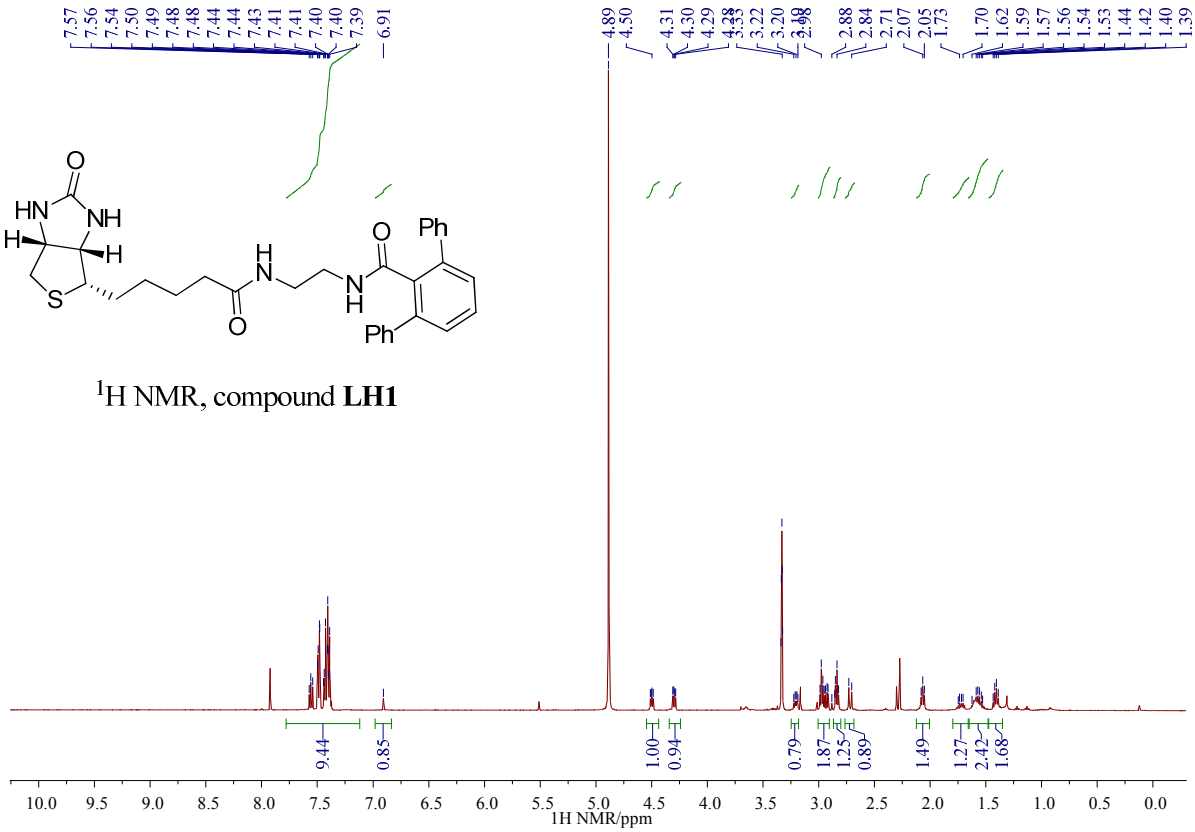
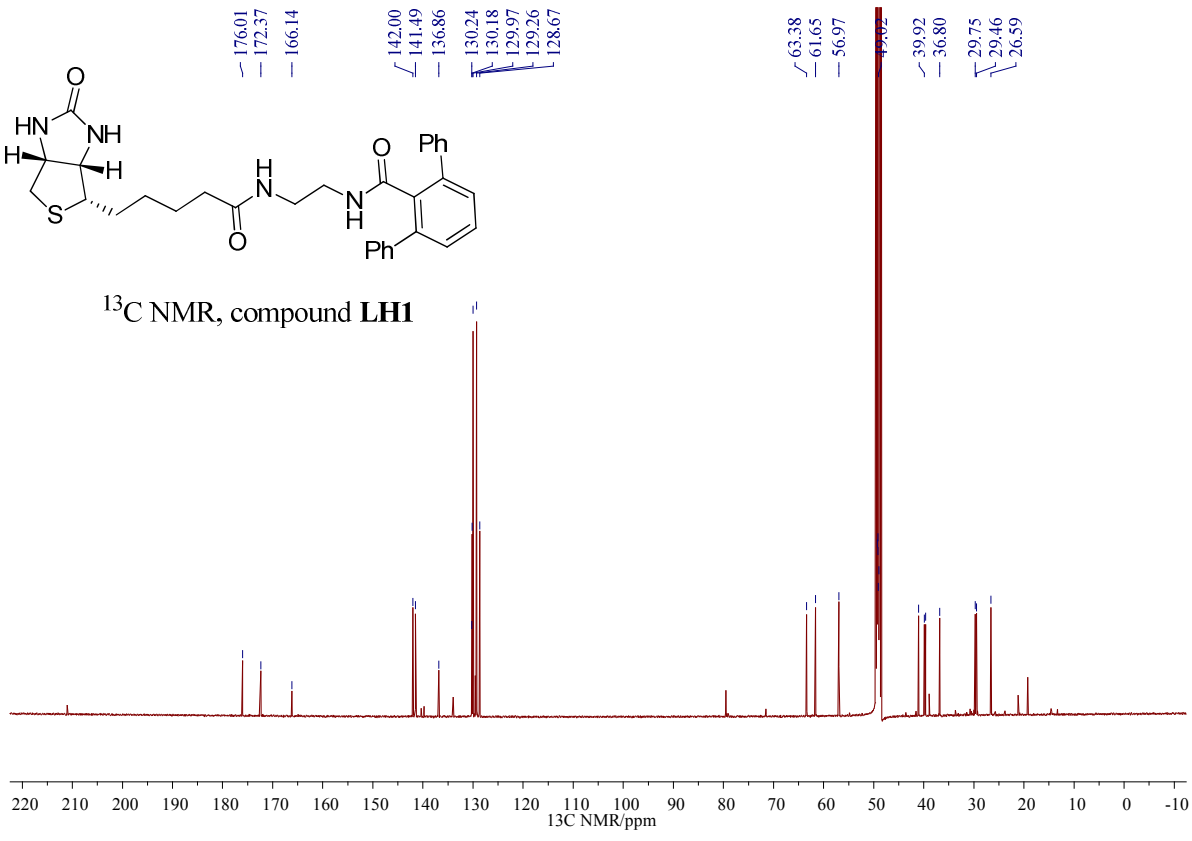


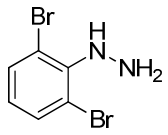
^{13}C NMR, compound **4**



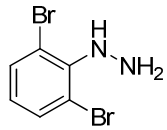
^{1}H NMR, compound **LH1**



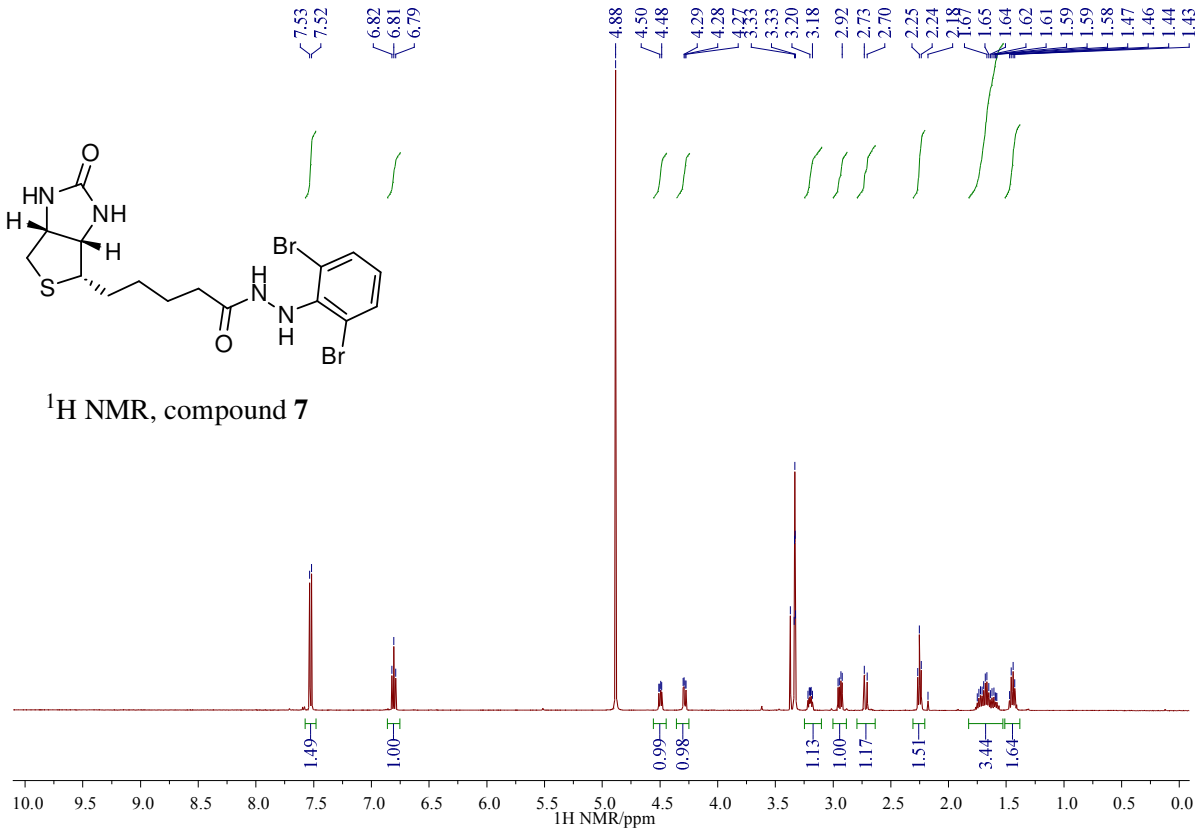
^{13}C NMR, compound **LH1**



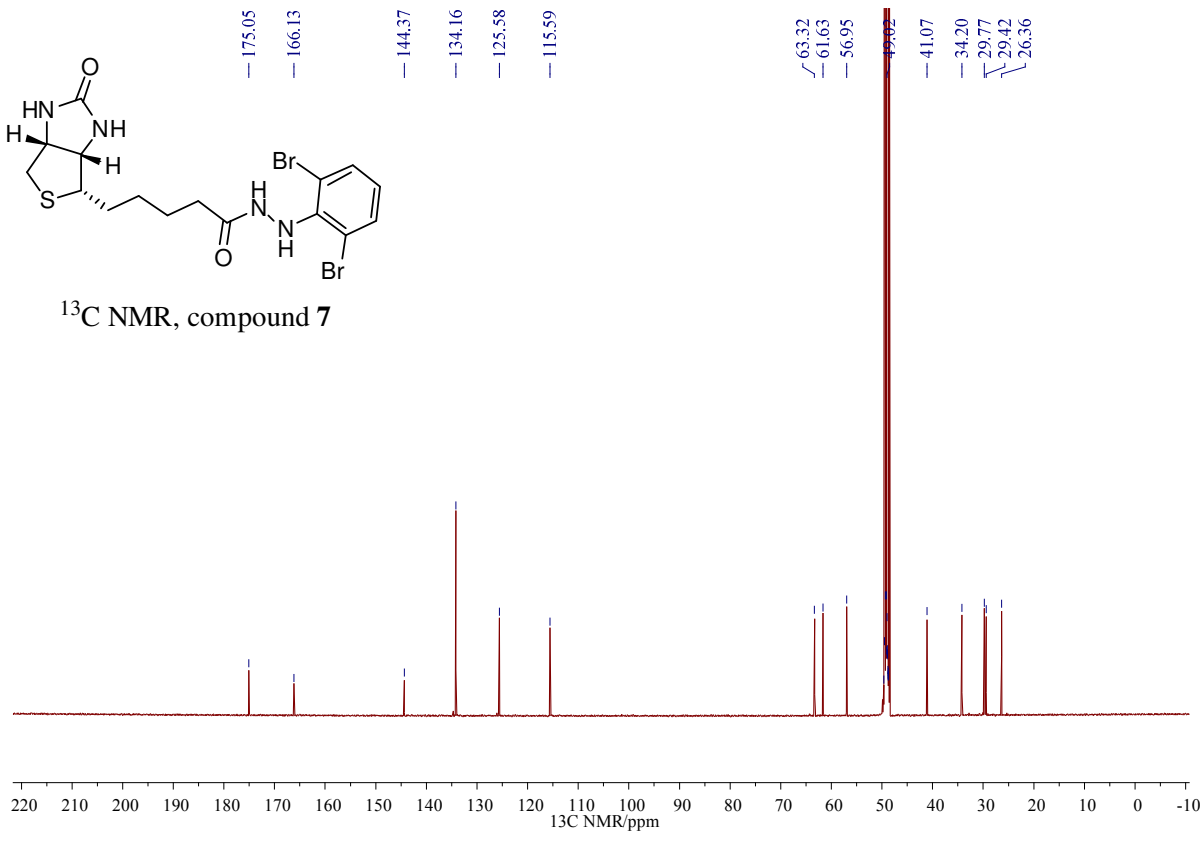
^{1}H NMR, compound **6**



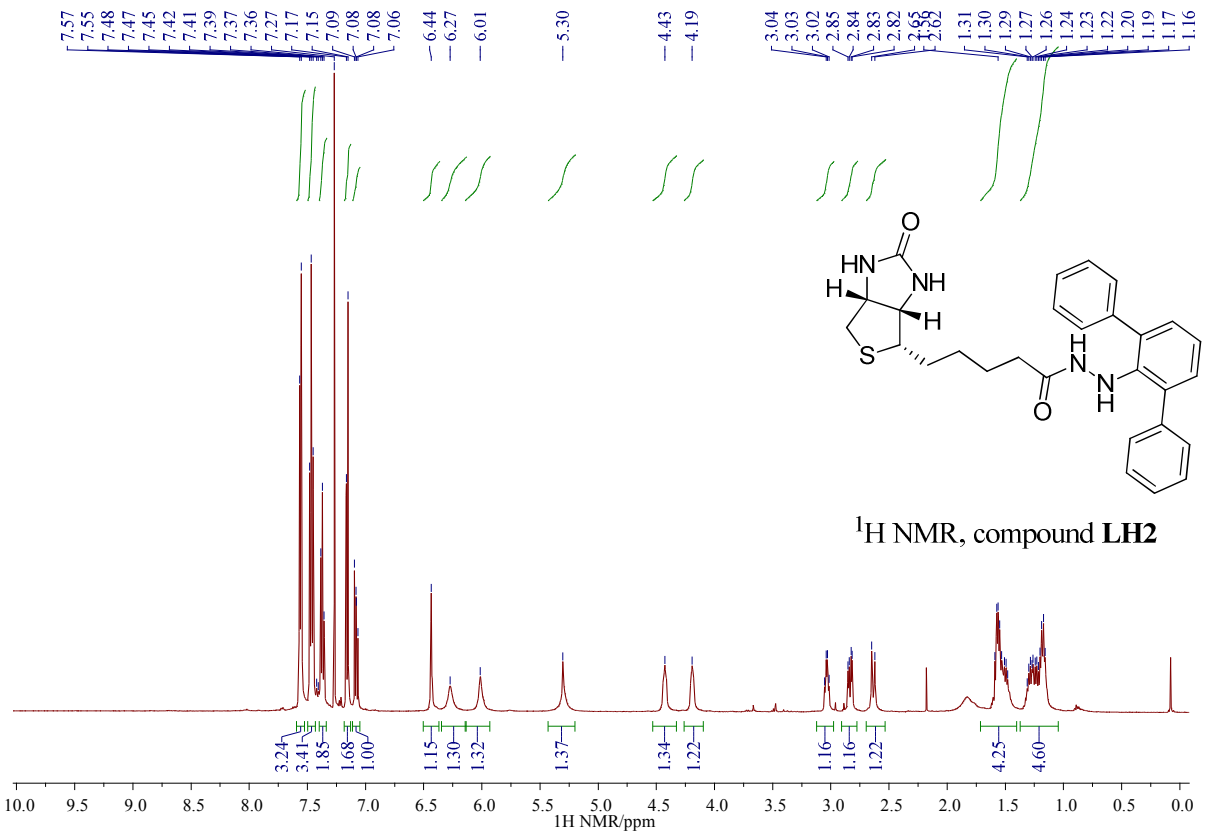
^{13}C NMR, compound **6**



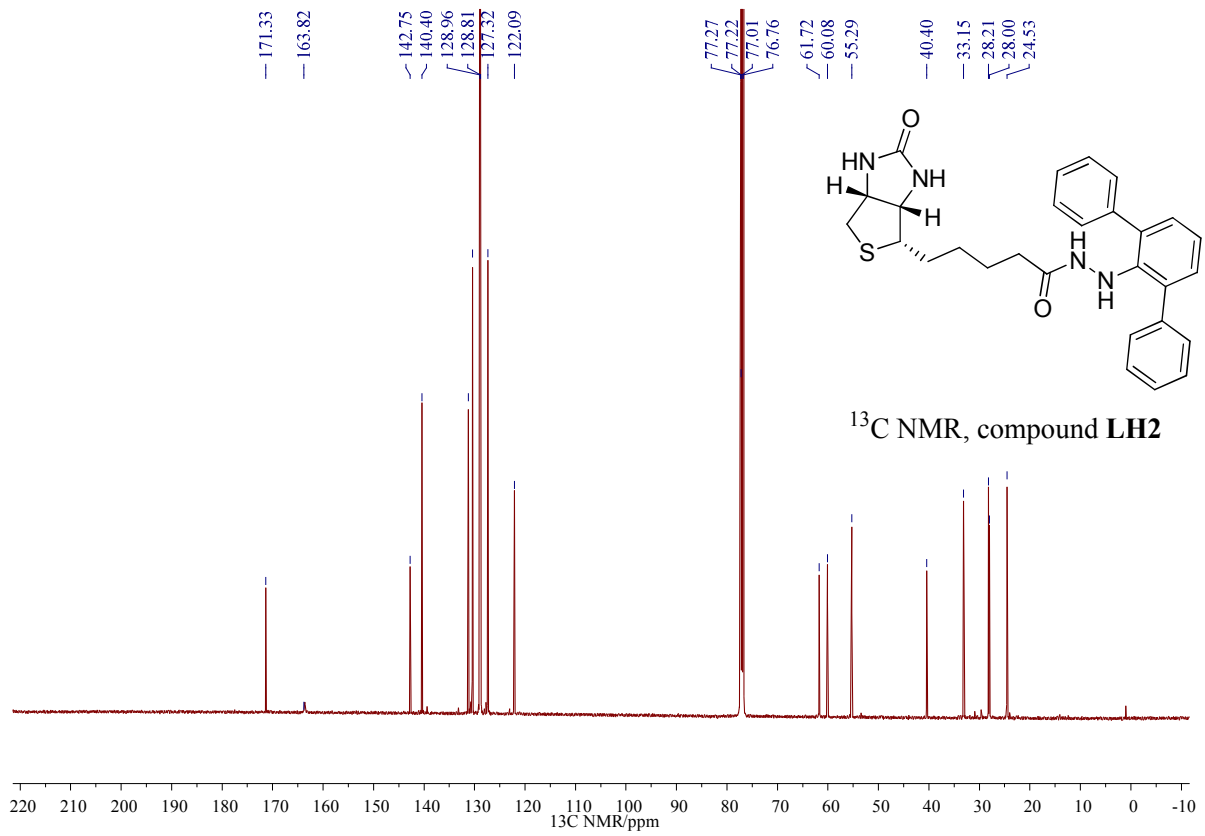
^{1}H NMR, compound **7**



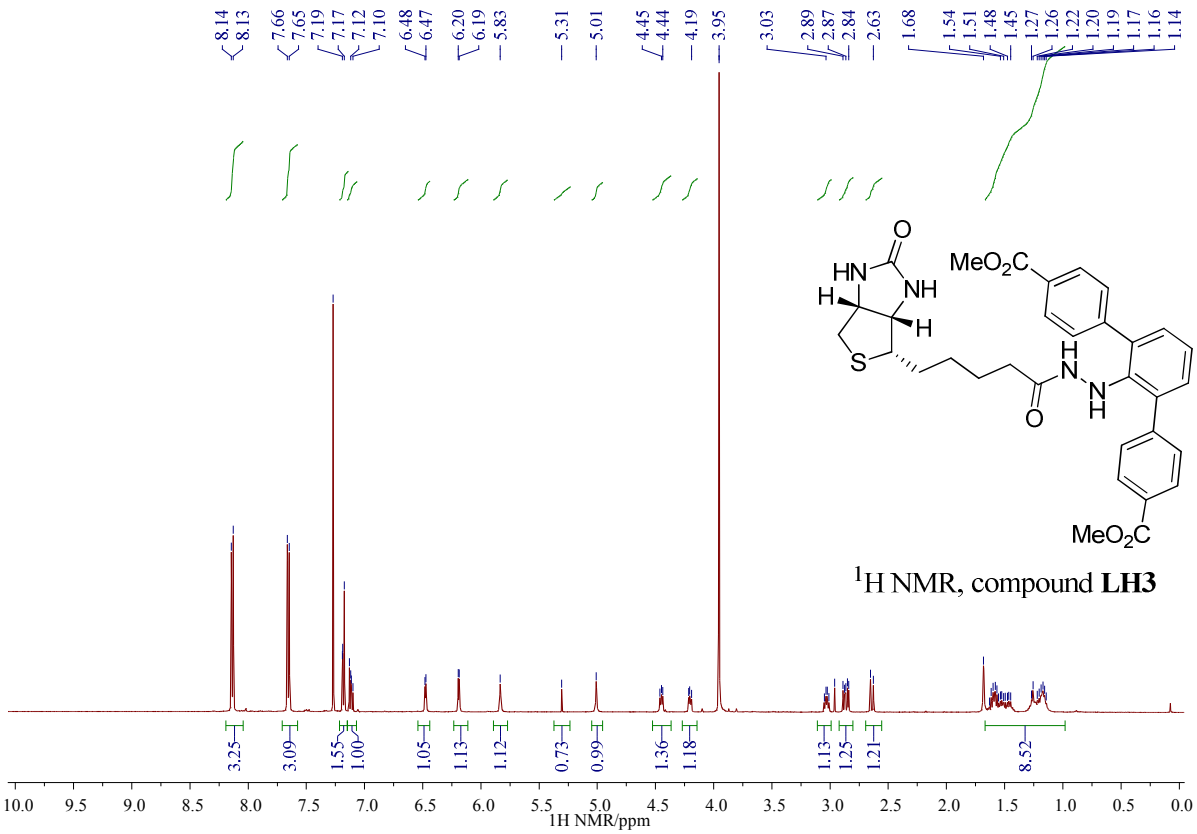
^{13}C NMR, compound **7**



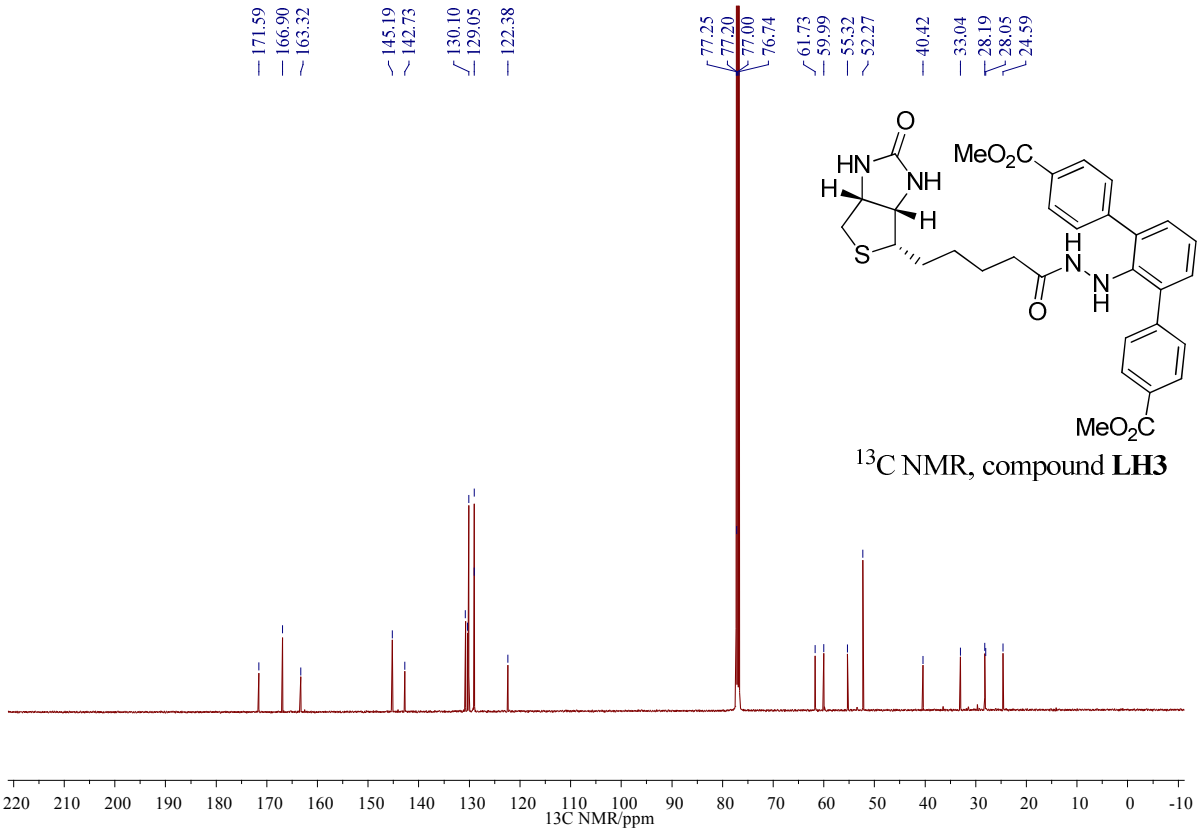
^{1}H NMR, compound **LH2**



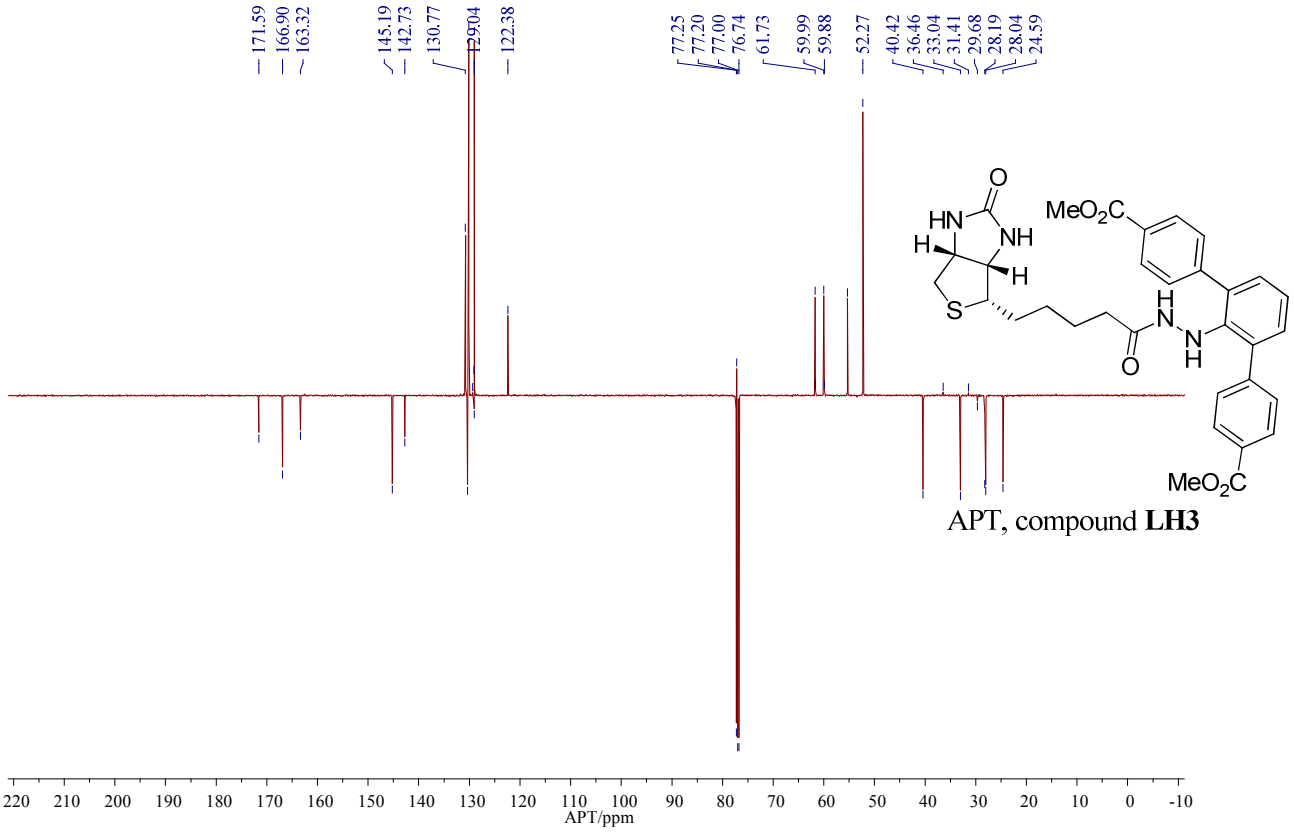
^{13}C NMR, compound **LH2**



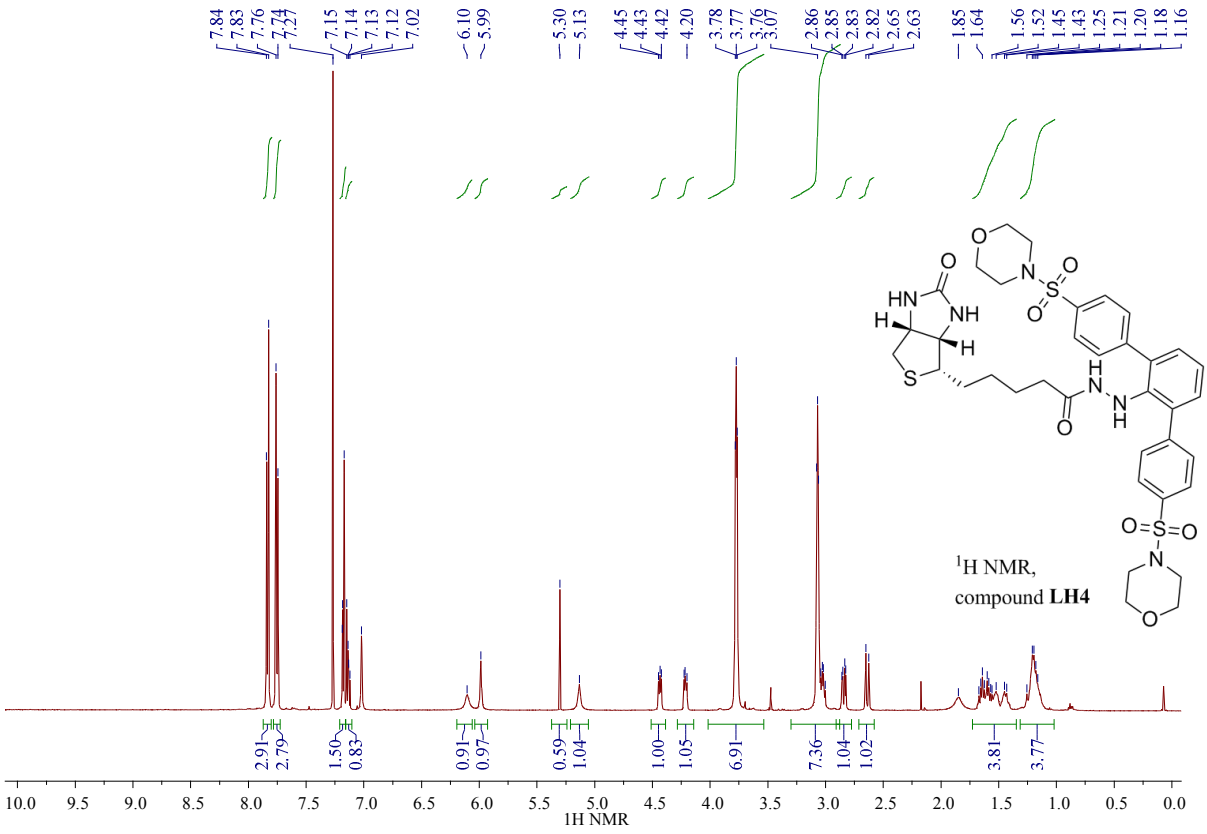
^{1}H NMR, compound **LH3**



^{13}C NMR, compound **LH3**



APT, compound **LH3**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5.13
4.45
4.43
4.42
4.20
3.78
3.77
3.76
3.07
2.86
2.85
2.83
2.82
2.65
2.63
1.85
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1.56
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1.20
1.18
1.16
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1.50
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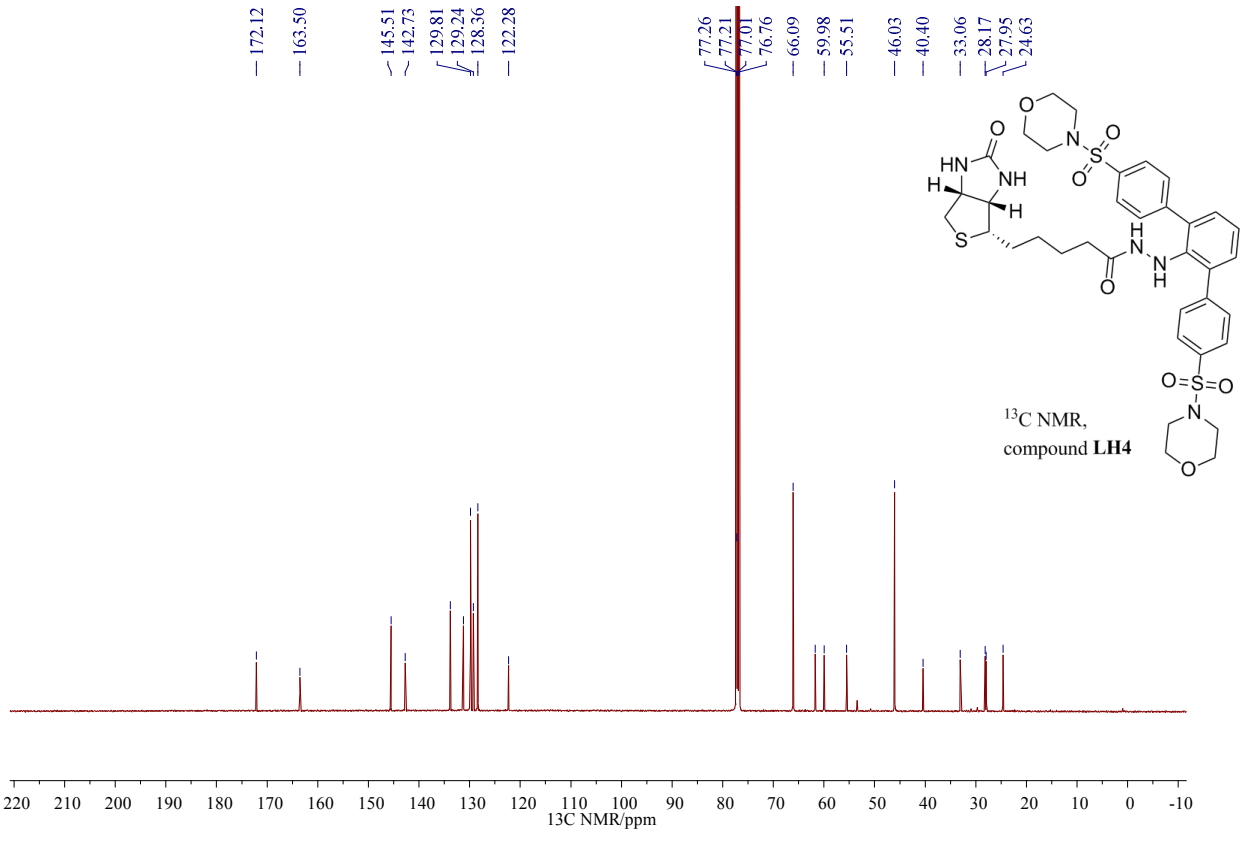
0.91
0.97
0.59
1.04
1.00
1.05
6.91
7.36
1.04
1.02
3.81
3.77
10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
1H NMR
^{1}H NMR,
compound **LH4**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46.03
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33.06
28.17
27.95
24.63
220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
13C NMR/ppm
^{13}C NMR,
compound **LH4**