Copper-free click enabled triazabutadiene for biorthogonal protein functionalization

Supporting Information

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General Information

Synthetic & Characterization

The chemicals including 4-aminobenzoic acid, conc. HCl, sodium nitrite, sodium azide, thionyl chloride, potassium *tert*-butoxide, 1-*tert*-butyl imidazole, methyl iodide, LiOH, diisopropylethylamine, *N*-hydroxysuccinimide, HATU, triethyl amine, D-biotin, 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and all the solvents and chromatography materials including silica and basic alumina were purchased commercially and used as received. 2-(2-(2-azidoethoxy)ethoxy)ethan-1-amine (CAS# 166388-57-4) was purchased from Lumiprobe and Dibenzocyclooctyne amine (DBCO-NH_2) was purchased from Click Chemistry Tools (Catalog number: A103-25).

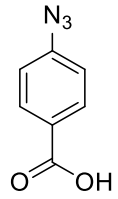
NMR spectra were taken either on a Bruker AVANCE III 400 MHz NMR, Bruker AVANCE DRX 500 MHz NMR or Bruker NEO 500 MHz NMR Spectrometer for ^{1}H and ^{13}C NMR and referenced with residual solvent peaks at 7.26 ppm and 77.0 ppm for $CDCl_3$, 2.50 and 39.5 for DMSO-d_6. Coupling on the NMR spectra is expressed in Hertz (Hz), chemical shifts in ppm, with abbreviations for multiplicities as s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, m = multiplet. Mass spectra were obtained using solariX 2XR 9.4T FTICR instrument. Mass spectral analysis was performed on a Bruker ICR ESI. Infrared spectra were obtained using a Thermo Fisher Scientific Nicolet IS50R FT-IR Spectrometer. The transmission mode was attenuated total reflectance (ATR), using a Spectra-Tech Thunderdome germanium crystal ATR accessory. The detector was a liquid nitrogen cooled high sensitivity MCT-A detector. The FT-IR signals are reported as w = weak, m = medium s = strong, br = broad.

Biochemical

Buffers were made appropriately using commercially purchased salts and nanopure water. They were checked for proper pH prior to experimental use. BSA stocks were made from commercially purchased lyophilized powder (Sigma Aldrich, CAS: 9048-46-8). MSP1D1T2(-) protein was expressed and purified by methods previously described.[1] Modified protein samples and controls were analyzed by SDS-PAGE on a 12% acrylamide resolving gel. Samples were treated with 1:1 ratio of 2X Laemmli sample buffer from Sigma Aldrich (Item S3401) containing 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, and 0.125 M Tris HCl pH 6.8. Alexa fluor™ 488 azide was purchased from Thermo-Fisher Scientific (Catalog number: A10266). Fluorescent imaging was performed with a ChemiDoc™ MP scanner (BioRad) using a 488 nm laser line (Pharos FX Plus Molecular Imager) with a 535 nm filter. Coomassie staining was imaged using the same scanner.

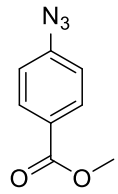
Synthetic Procedures and Characterization

4-azido benzoic acid - (SI-1)



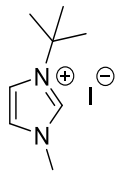
SI-1 was synthesized as an off-white solid (0.546 g, 93 %) according to the previously reported methods.[2] **^{1}H NMR** (400 MHz, DMSO-d_6) δ 12.94 (s, 1H), 7.95 (d, J = 8.1 Hz, 2H), 7.21 (d, J = 8.2 Hz, 2H). **^{13}C NMR** (100 MHz, DMSO-d_6) δ 166.53, 143.92, 131.19, 127.27, 119.15.

methyl 4-azidobenzoate - (2)



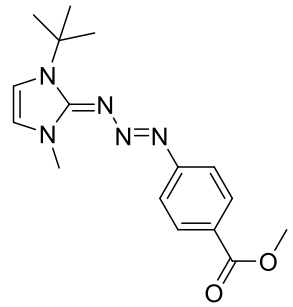
2 was synthesized as a brownish yellow crystalline solid (0.280 g, 88 %) according to the previously reported methods.[2] **^{1}H NMR** (400 MHz, DMSO-d_6) δ 8.02 – 7.91 (m, 2H), 7.32 – 7.20 (m, 2H), 3.84 (s, 3H). **^{13}C NMR** (100 MHz, DMSO-d_6) δ 165.45, 144.38, 131.04, 126.02, 119.33, 52.10.

3-(*tert*-butyl)-1-methyl-1*H*-imidazol-3-ium iodide- (3)



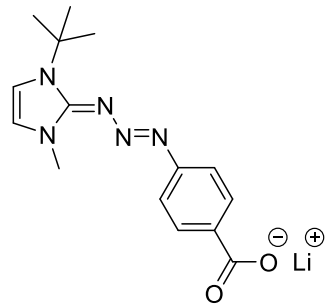
t-butyl imidazole (0.124 g, 1.00 mmol) was dissolved in 2 mL of acetonitrile. Methyl iodide (0.178 g, 1.25 mmol) was added to this dropwise at 0 °C. The reaction was stirred overnight at RT. The solvent was evaporated under vacuum and the product (**3**) was isolated as a white powder in quantitative yields. The above procedure was a slight modification of an existing procedure.[3] **^{1}H NMR** (400 MHz, Chloroform-d) δ 9.97 (q, J = 1.4 Hz, 1H), 7.58 (t, J = 1.9 Hz, 1H), 7.51 (t, J = 1.9 Hz, 1H), 4.11 (s, 3H), 1.67 (s, 9H). **^{13}C NMR** (100 MHz, Chloroform-d) δ 135.20, 123.85, 119.48, 60.39, 36.91, 30.04.

Methyl 4-((*E*)((*E*)-1-(*tert*-butyl)3-methyl-1,3-dihydro-2*H*-imidazol-2-ylidene)triaz-1-en-1-yl)benzoate - (SI-2)



In a flame-dried round bottom flask, azide **2** (0.450 g, 2.54 mmol, 1 equiv) and imidazolium **3** (0.798 g, 3.00 mmol, 1.2 equiv) were dissolved in anhydrous THF (10 mL). To the solution was added *t*-BuOK (0.560 g, 5.00 mmol, 2 equiv) in one portion at 0 °C while stirring. The reaction was allowed to warm to RT slowly removing the ice bath and stirred for 3 h. The solution was filtered using a filter paper, washing with CH_2Cl_2 and the solvent was evaporated to provide a crude product. The mixture was purified using a basic alumina column (Hexanes : Ethyl Acetate = 1 : 2, R_f = 0.3) to afford the product (**SI-2**) as a bright yellow solid (0.607 g, 77 %). **^{1}H NMR** (400 MHz, DMSO-d_6) δ 7.91 – 7.84 (m, 2H), 7.45 – 7.36 (m, 2H), 7.18 (d, *J* = 2.6 Hz, 1H), 7.03 (d, *J* = 2.5 Hz, 1H), 3.82 (s, 3H), 3.75 (s, 3H), 1.62 (s, 9H). **^{13}C NMR** (100 MHz, DMSO-d_6) δ 166.19, 156.90, 151.03, 130.16, 124.42, 119.82, 118.21, 113.45, 58.09, 51.73, 38.46, 28.27. **IR** (ATR) 1708 (m), 1597 (w), 1521 (m), 1347 (m), 1268 (m), 1196 (m), 1150 (s), 1113 (m), 1099 (w), 851 (w), 773 (m). **HRMS** (ESI) m/z: [M + H]+ calculated for $C_{16}H_{21}N_5O_2$ 316.1773; found value 316.1765.

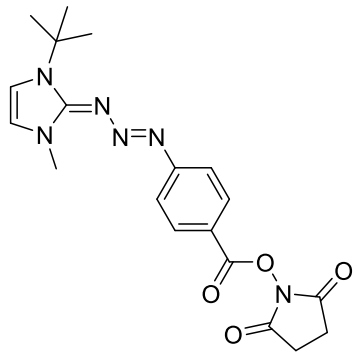
4-((*E*)-((*E*)-1-(*tert*-butyl)-3-methyl-1,3-dihydro-2*H*-imidazol-2-ylidene)triaz-1-en-1-yl)benzoate lithium (I) - (SI-3)



To methyl ester (**SI-2**) (60 mg, 0.19 mmol, 1 equiv) charged in a round bottom flask was added a 10 % LiOH suspension in water (10 mg, 100 µL, 0.42 mmol, 2.2 equiv). A mixture of 5 mL of THF and 5 mL of DI H_2O was added to the mixture. The reaction was heated to 80 °C in an oil bath while stirring vigorously. After 2 h the reaction was stopped removing from the oil bath, and the organic layer was evaporated using a vacuum pump. Remaining water was removed by blowing air on the reaction mixture. The crude product mixture (**SI-3**) was clean enough and carried on to the next step without further purification.

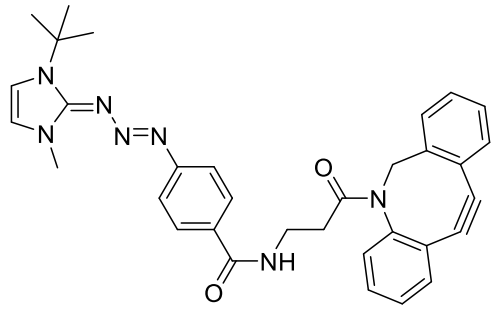
^{1}H NMR (500 MHz, DMSO-d_6) δ 7.83 – 7.80 (m, 2H), 7.25 – 7.21 (m, 2H), 7.08 (d, *J* = 2.6 Hz, 1H), 6.92 (d, *J* = 2.6 Hz, 1H), 3.73 (s, 3H), 1.61 (s, 9H).**^{13}C NMR** (126 MHz, DMSO-d_6) δ 169.38, 152.93, 151.61, 136.91, 129.76, 118.79, 117.62, 112.75, 57.58, 38.60, 28.18. **IR** (ATR) 1596 (m), 1557 (w), 1524 (m), 1453 (w), 1398 (m), 1353 (m), 1277 (m), 1244 (w), 1233 (w), 1192 (s), 1162 (m), 1100 (w), 869 (w), 844 (w), 834 (w), 818 (w), 795 (m), 775 (w), 755 (w), 750 (w). **HRMS** (ESI) m/z: [M + 2H]+ calculated for $C_{15}H_{18}N_5O_2^-$ 302.1617; found value 302.1613.

2,5-dioxopyrrolidin-1-yl 4-((*E*)-((*E*)-1-(*tert*-butyl)-3-methyl-1,3-dihydro-2*H*-imidazol-2-ylidene)triaz-1-en-1-yl)benzoate - (4)



The crude **SI-3** (all from previous experiment) was dissolved in anhydrous CH_2Cl_2 (20 mL) sonicating if needed. To this mixture was added a small amount (30 mg) of anhydrous $MgSO_4$. Further, diisopropylethylamine (100 µL, 0.56 mmol, 2.9 equiv), *N*-hydroxysuccinimide (50 mg, 0.43 mmol, 2.3 equiv) and finally HATU (80 mg, 0.21 mmol, 1.1 equiv) were added in order. The reaction was stirred overnight (16 h). The reaction was filtered, evaporated and the product formation was confirmed by taking a crude ^{1}H NMR in DMSO. The NHS-ester, **4**, was carried on to the next step without further purification.**^{1}H NMR** (400 MHz, DMSO-d_6) δ 7.97 (d, 2H), 7.50 (d, 2H), 7.25 (d, 1H), 7.10 (d, 1H), 3.77 (s, 3H), 2.88 (s, 4H), 1.63 (s, 9H).

TBD-cyclooctyne (1)

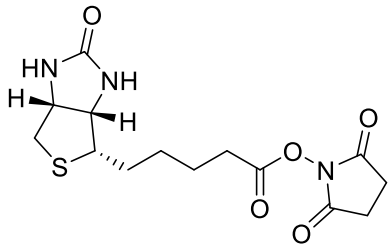


Assuming the complete conversion of **SI-2** to **4** based on the H NMR**,** a crude solution of TBD-NHS (**4**) (76 mg/mL, 0.19 M) was prepared in anhydrous CH_2Cl_2. To a flame dried round bottomed flask was added DBCO-NH_2 (12.6 mg, 0.045 mmol, 1 equiv) dissolved in 3 mL of anhydrous CH_2Cl_2. To this was added triethyl amine (5.4 µL, 0.045 mmol, 1 equiv) and a small amount (30 mg) of anhydrous $MgSO_4$. To this mixture, added was (237 µL, 0.045 mmol, 1 equiv) of the 76 mg/mL solution of **4**. The reaction was stirred 16 hours and then filtered, washing with CH_2Cl_2, and evaporated under vacuum. The crude product was purified using a basic alumina column and a solvent gradient. CH_2Cl_2 : ether = 4 : 1 and CH_2Cl_2 : ether 1 : 1 was used to get rid of non - polar impurities as the product stuck on the baseline. Then 2% MeOH : CH_2Cl_2 was used, and the product came off at R_f = 0.3. However, this had 2 other UV active spots when stained with I_2. A trituration was done with CH_2Cl_2 and hexanes as an additional purification step. A yellow precipitate crashed out from the solution and was filtered to provide pure **1** (8.3 mg, 33% yield over 3 steps). The compound was stable in a solution of DMSO-d_6 for 5 months at 0 °C.

^{1}H NMR (500 MHz, DMSO-d_6) δ 8.11 (t, J = 5.6 Hz, 1H), 7.67 – 7.65 (m, 1H), 7.64 – 7.59 (m, 3H), 7.52 – 7.47 (m, 1H), 7.47 – 7.42 (m, 2H), 7.42 – 7.38 (m, 1H), 7.38 – 7.33 (m, 1H), 7.33 – 7.30 (m, 2H), 7.30 – 7.27 (m, 1H), 7.15 (d, J = 2.6 Hz, 1H), 7.00 (d, J = 2.5 Hz, 1H), 5.07 (d, J = 14.0 Hz, 1H), 3.74 (s, 3H), 3.64 (d, J = 14.0 Hz, 1H), 3.39 – 3.33 (m, 1H), 3.14-3.07 (m, 1H), 2.58-2.51 (m, 1H), 2.01-1.92 (m, 1H), 1.61 (s, 9H). **^{13}C NMR** (126 MHz, $CDCl_3$) δ 170.42, 166.03, 151.59, 151.31, 148.51, 132.53, 129.77, 129.63, 129.03,

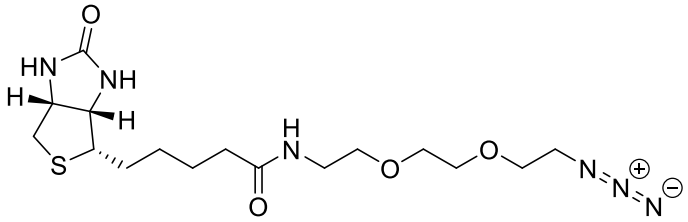
128.30, 128.15, 128.03, 127.80, 126.90, 125.30, 122.58, 121.49, 119.51, 118.15, 114.40, 113.35, 108.20, 57.96, 54.80, 38.46, 35.68, 34.25, 28.21. **IR** (ATR) 2157 (w), 1649 (m), 1601 (w), 1525 (m), 1483 (m), 1449 (w), 1414 (w), 1397 (w), 1371 (m), 1356 (m), 1323 (w), 1276 (s), 1245 (w), 1157 (s), 1105 (s), 1009 (w), 945 (w), 856 (w), 770 (m), 753 (m). **HRMS** (ESI) m/z: [M + H]+ calculated for $C_{33}H_{33}N_7O_2$ 560.2774; found value 560.2755.

2,5-dioxopyrrolidin-1-yl 5-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanoate (SI-4)



SI-4 was synthesized as a white solid (0.952 g, 93 %) according to the previously reported methods.[4] **^{1}H NMR** (400 MHz, DMSO-d_6) δ 6.42 (s, 1H), 6.36 (s, 1H), 4.30 (m, 1H), 4.14 (m, 1H), 3.10 (m, 1H), 2.91 – 2.82 (m, 1H), 2.81 (s, 4H), 2.76 – 2.63 (m, 2H), 2.58 (d, *J* = 12.4 Hz, 1H), 1.64 (m, 3H), 1.57 – 1.35 (m, 3H). **^{13}C NMR** (100 MHz, DMSO-d_6) δ 170.24, 168.91, 162.66, 60.98, 59.15, 55.21, 29.98, 27.82, 27.56, 25.43, 24.29.

***N*-(2-(2-(2-azidoethoxy)ethoxy)ethyl)-5-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamide (SI-5)**



A modification of an existing protocol was followed.[5] To biotin-NHS (**SI-4**) (50 mg, 0.15 mmol, 1 equiv) dissolved in dry DMF (5 mL), added was triethyl amine (40 µL, 0.29 mmol, 1.9 equiv) and commercially available 2-(2-(2-azidoethoxy)ethoxy)ethan-1-amine (38.33 mg, 0.22 mmol, 1.5 eq). The reaction was stirred overnight (16 h) and the solvent was evaporated. The resulting crude mixture was re-dissolved in CH_2Cl_2 and treated with aqueous $NaHCO_3$. Next, the organic layer washed with brine, dried over $MgSO_4$ and evaporated under vacuum. Pure compound (**SI-5**) was obtained as a yellow glassy solid in appearance. (0.028 mg, 47%). The spectral data matched with literature.[6]

^{1}H NMR (400 MHz, DMSO-d_6) δ 7.80 (t, *J* = 5.6 Hz, 1H), 6.41 (s, 1H), 6.34 (s, 1H), 4.32 – 4.27 (m, 1H), 4.15 - 4.10 (m, 1H), 3.61 - 3.58 (m, 2H), 3.58 - 3.54 (m, 2H), 3.54 - 3.50 (m, 2H), 3.42 – 3.37 (m, 4H), 3.21 - 3.16 (m, 2H), 3.12 – 3.06 (m, 1H), 2.84 – 2.79 (m, 1H), 2.60 – 2.55 (d, *J* = 12.4 Hz, 1H), 2.08 – 2.03 (t, *J* = 7.4 Hz, 2H), 1.66 – 1.55 (m, 1H), 1.55 – 1.43 (m, 3H), 1.33 – 1.24 (m, 2H). **^{13}C NMR** (100 MHz, DMSO-d_6) δ 172.07, 162.66, 69.59, 69.54, 69.23, 69.13, 61.01, 59.16, 55.39, 49.97,39.82, 38.40, 35.07, 28.16, 28.01, 25.23.

Biological Studies and Protocols

*BSA labeling with **1** at various pH (4-9)*

In this experiment various buffers were used to preserve pH. For the pH 4-5 range, 0.1 M citrate was used. For samples pH 6-8, 0.1 M PBS was used. 5 µL of 1 mM BSA was transferred to 172.8 µL of respective buffer (pH 4-8). Two samples were made for each pH. The first sample of each set was a control sample treated with 22.2 µL of DMSO (~11% by volume). The second sample for each pH unit was treated with 22.2 µL of 1.8 mM **1**. The final concentration of BSA in each sample was 25 µM and the final concentration of **1** was 0.2 mM. All samples were incubated void of light for 3 hours and then treated with 20 µL of 100 mM resorcinol to quench the remaining aryl diazonium ion (final concentration of 9 mM resorcinol). 30 µL of each sample was then transferred to a new Eppendorf and treated with 1.2 µL of 5 mM Alexa Fluor azide to provide a concentration of 0.2 mM. Samples were further incubated void of light for 2 hours prior to being loaded on an SDS-PAGE.

*Kinetic evaluation of BSA labeling with **1** at pH 7*

10 µL of 1 mM BSA was diluted into 383.7 µL of 0.1 M PBS buffer (pH 7) in a 1.6 mL Eppendorf tube. Next, 6.35 µL of 12.6 mM **1** was added to the solution to provide a final concentration of 25 µM BSA and 0.4 mM of **1**. Upon addition, aliquots of the reaction solution were removed at various time points of 5 min, 15 min, 30 min, 1 hour, 2 hour, 4 hours and 6 hours. In addition, a second sample of equal BSA concentration was preincubated with 20 mM resorcinol prior to the addition of **1** to act as the 0 min time point. Upon reaching each time point, 50 µL of the reaction sample was transferred to a separate Eppendorf and treated with 2 µL of 0.5 M resorcinol to quench the diazonium reaction, providing a final resorcinol concentration of ~19.2 mM. After the final time point, 10 µL of each sample was removed to separate Eppendorf tubes and treated with 0.5 µL of 5 mM Alexa Fluor azide (Thermo-Fisher Scientific; Catalog number: A10266) to provide a final concentration of 0.4 mM. The click reaction proceeded for 2 hours prior to analysis on SDS-PAGE.

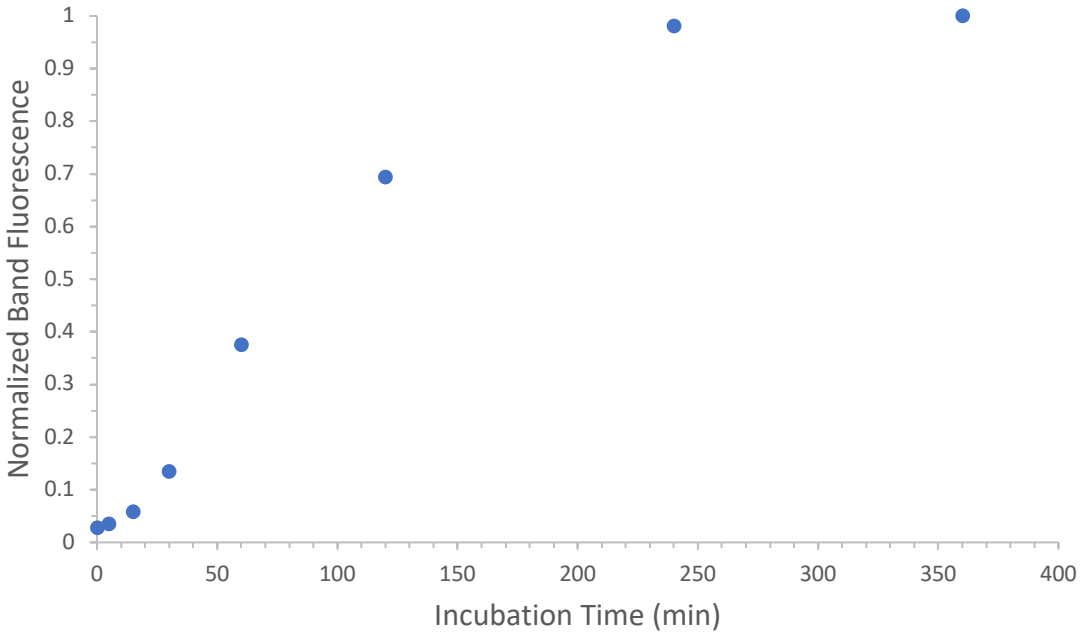


Figure S1. BSA treatment with 0.4 mM **1** across time points of 0 to 360 minutes. Following SDS-PAGE analysis, band fluorescence was quantified by ImageJ software and normalized to the Coomassie band density for each corresponding band. Normalized fluorescence was plotted as a function of time showing that after about 240 minutes (4 hours), fluorescence maxes out, indicating that the TBD has finished reacting.

Quantification of gel band fluorescence with ImageJ Software for kinetic evaluation

Using ImageJ software, the band intensities for both fluorescence and Coomassie scans were determined for each sample using the band integration tool. First, band fluorescence was normalized to the band with the highest fluorescence intensity amongst all samples by dividing all by its respective integration value. Next, we integrated the band intensity of the corresponding Coomassie stain. We then normalized the Coomassie band intensity to the highest band intensity providing a ratio between 0-1. Lastly, each band fluorescence was divided by its respective Coomassie ration to normalize fluorescence intensity to the relative quantity of protein per band. This was done to accommodate for slight differences in protein concentrations between wells. Normalized fluorescent intensities were then plotted as a function of Time (**SF1**).

*BSA labeling with **1** with resorcinol competition*

In two Eppendorf tubes, 1 mM BSA was diluted to 50 µM in 0.1 M PBS buffer (pH 7). To the experimental sample, 10 µL of 0.5 M resorcinol stock was added to provide a final concentration of 20 mM. Both samples were then treated with 8 µL of 12.6 mM **1** (0.4 mM) in a total volume of 250 µL. Samples were incubated void of light overnight (~16 hours). 10 µL of each sample was treated with 1 µL of 5 mM Alexa Fluor azide to provide a final concentration of 0.5 mM. Click reaction proceeded for 2 hours prior to analysis on SDS-PAGE.

*Reduction of **1** labeled BSA with sodium dithionite*

1 mM BSA was diluted to 50 µM in 0.1 M PBS buffer (pH 7). To this sample was added 8 µL of 12.6 mM **1** (0.4 mM) in a total volume of 250 µL. The protein solution was left to react void of light overnight (~16 hours). Next, the sample was split into two 125 µL aliquots. The experimental sample was then treated with 6.25 µL of 1 M sodium dithionite to provide a final concentration of 50 mM. In the control sample, 6.25 µL of buffer was added. The reduction reaction was incubated for 2 hours. Samples were then passed through a Size Exclusion Column with G-10 resin. Following buffer exchange, 10 µL of each sample was treated with 1 µL of 5 mM Alexa Fluor azide to provide a final concentration of 0.5 mM. The click reaction proceeded for 2 hours prior to analysis on SDS-PAGE.

*Treatment of BSA with **1** in the presence of excess cysteine*

In a 1.6 mL Eppendorf, 1.25 µL of 1 mM BSA was diluted into 0.1 M PBS buffer (pH 7) to provide a final concentration of 25 µM protein. Sample one, the positive control sample, was treated with 1.1 µL of a 12.6 mM stock of **1** dissolved in DMSO to provide a final concentration of 0.5 mM **1** in solution in a total volume of 50 µL. An analogous experimental sample was then made with 25 µM BSA in 0.1 M PBS (pH 7). Prior to addition of **1**, the sample was first treated with 2 µL of a 0.5 M stock of cysteine to provide a final cysteine concentration of 20 mM. The sample was subsequently treated with 0.5 mM **1** and incubated overnight. 10 µL of each sample was then treated with 0.5 µL of 5 mM Alexa Fluor azide for 4 hours. Samples were compared on 12% SDS-PAGE via scanning for fluorescence at 488 nm.

Treatment of DBCO-modified BSA with excess cysteine

25 µM BSA was treated with 0.5 mM **1** overnight and then the sample was treated with 20 mM resorcinol to quench potential diazonium reactivity. Next, the sample was split into two 50 µL aliquots. The second aliquot was then treated with 20 mM cysteine and let incubate for ~16 hours overnight. 10 µL of each sample was then treated with 5 mM Alexa Fluor azide for 4 hours. Samples were compared on 12% SDS-PAGE via scanning for fluorescence at 488 nm.

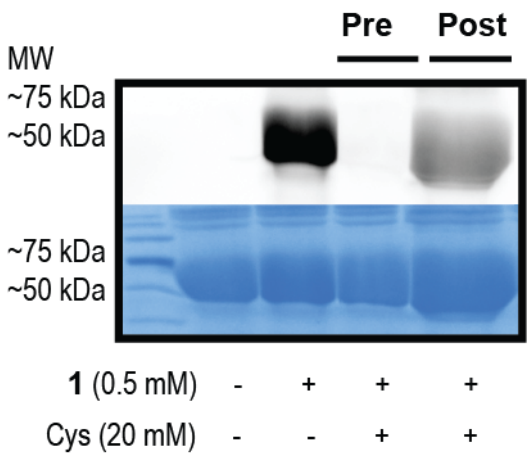


Figure S2. BSA treatment with 0.5 mM **1** was performed overnight (Lane 2). A second sample was treated first with 20 mM cysteine prior to addition of **1** (Lane 3). The final sample took the BSA treated with **1** overnight and then further treated it with 20 mM cysteine following an addition of resorcinol used to quench any remaining diazonium (Lane 4). Fluorescence was completely negated when cysteine was present during the initial diazonium conjugation. However, fluorescence remained when cysteine was introduced after conjugation indicating the cyclooctyne was mostly stable to thiol-yne reactivity.

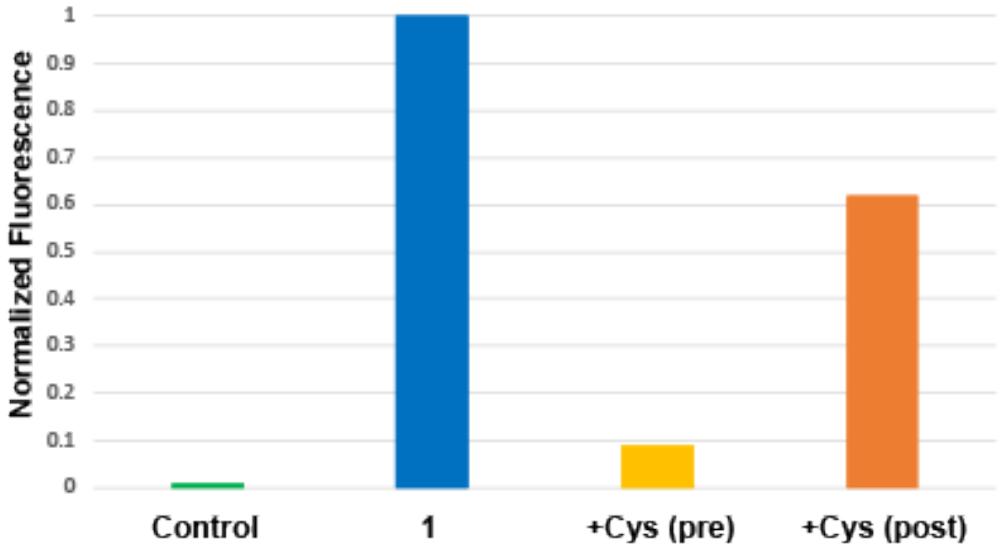


Figure S3. Quantification of normalized band fluorescence for control sample (green), BSA treated with 0.5 mM **1** (blue), BSA treated with 0.5 mM **1** in the presence of 20 mM cysteine (yellow), and BSA modified by **1** subsequently treated with 20 mM cysteine (orange).

Quantification of gel band fluorescence with ImageJ Software after cysteine treatment

Using ImageJ software, band intensities for both fluorescence and Coomassie scans were determined for each sample using the band integration tool. First, band fluorescence was normalized to the band with the highest fluorescence intensity amongst all samples by dividing all by its respective integration value. Next, we integrated the band intensity of the corresponding Coomassie stain. We then normalized the Coomassie band intensity to the highest band intensity providing a ratio between 0-1. Lastly, each band fluorescence was divided by its respective Coomassie ration to normalize fluorescence intensity to the relative quantity of protein per band. This was done to accommodate for slight differences in protein concentrations between wells. Normalized fluorescent intensities were then plotted on a bar chart showing relative fluorescence on a scale from 0 to 1 (**SF3**).

*SDS-PAGE analysis of MSP1D1T2(-) labeling with **1** and AlexaFluor azide*

For initial determination of labeling of MSP by **1**, we chose to treat MSP1 with **1** and then subsequently treated the sample with Alexa Fluor azide (488 nm) in order to be analyzed for fluorescence on SDS-PAGE. 10 µL of a 25 µM solution of MSP previously treated with 0.5 mM of **1** was subsequently treated with 0.25 mM of the fluorescent azide via addition of 1.25 µL of a 5 mM stock of Alexa Fluor azide in DMSO. This sample was compared to an analogous protein sample containing equal concentration (25 µM) of MSP and treated with 0.25 mM azide, but not previously treated with **1**. Samples were incubated at RT for 1 hour prior to loading on 12% SDS-PAGE gel and analyzed using 488 nm laser line.

*Labeling of MSP1D1T2(-) with **1** and PEG_2 – Biotin Azide*

An initial stock of 150 µM MSP was provided to us following expression and purification previously described.[1] The stock was used to make 2 samples consisting of MSP in 0.1 M PBS buffer pH 7. For the non-modified control, 16.7 µL of the protein was added directly to 83.3 µL of PBS buffer pH 7 and incubated (25 µM protein). For the TBD treated sample, the volume was increased to 500 µL to allow for aliquots. 83.5 µL of protein was added to 396.5 µL of PBS buffer pH 7 to provide a final protein concentration of 25 µM protein. The sample was then treated with 20 µL of 12.6 mM **1** to provide a concentration of 0.5 mM of **1**. The samples were then incubated overnight (~14 hrs). Following prolonged incubation, the **1** treated sample was split into two aliquots of 200 µL. Both aliquots were treated with 100 mM resorcinol prior to further additions in order to quench and possible remaining diazonium activity. The first aliquot was not treated further. The second aliquot was then treated with PEG_2 – Biotin azide. First, a 20 mM stock of the biotin azide was made in DMSO by dissolving 8 mg in 1 mL of DMSO. Next, 5 µL of the stock was added to the 200 µL **1** treated sample to provide a final concentration of 0.49 mM (~1 equivalent) of azido biotin relative to **1**. The sample was further incubated at room temperature for 4 hours before being placed in the -20 ^{o}C freezer until the sample could be prepped for Native MS.

MSP1D1T2(-) sample analysis by Native Mass Spectrometry

Each sample was buffer exchanged twice into 0.2 M ammonium acetate (pH 6.8) using a Micro Bio-spin P-6 column (BioRad). The final concentration of each sample prior to analysis was ~65 µM. Native mass spectrometry (MS) was performed using the Q-Exactive HF quadrupole-Orbitrap mass spectrometer with Ultra-High Mass Range modifications (Thermo Fisher Scientific). Native mass spectrometry was performed in positive-ion mode using borosilicate nano-electrospray ionization needles pulled using a P-1000 micropipette puller (Sutter Instrument, Novato, CA). Instrumental parameters applied include 1.1 kV spray voltage, 50 V source voltage, and 50 V collisional voltage. The instrument had a trapping gas pressure set to 3. The scan range for all samples was 1,500-15,000 *m/z,* and the instrumental resolution was set to 15,000. Data was analyzed with UniDec.[7]

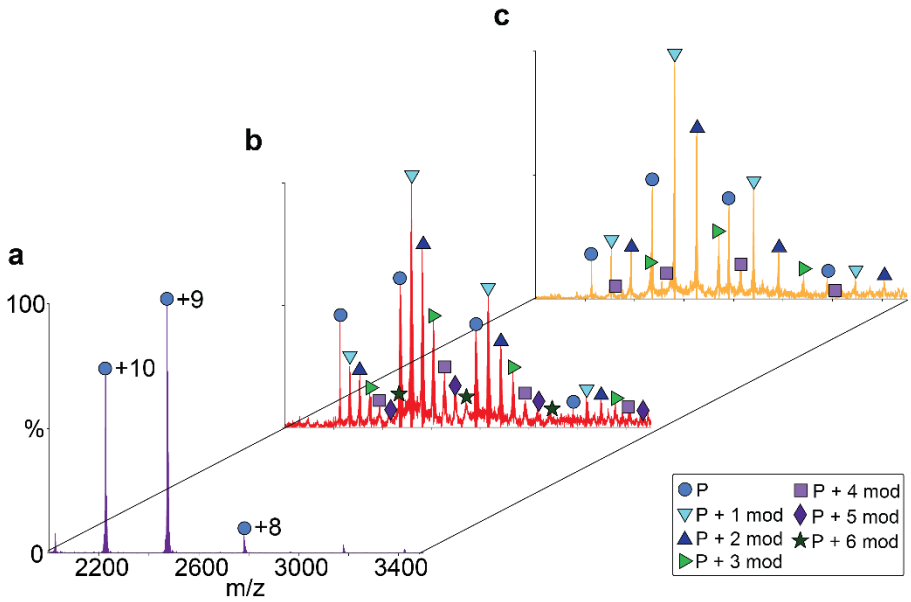


Figure S4. *m*/z data collected for comparison of MSP1D1T2(-) (MSP) before and after modification with **1** and subsequent copper-free click functionalization (**a**) Native MS *m*/z data for MSP control (purple). (**b**) Native MS *m*/z data for MSP treated with 0.5 mM **1** (red). (**c**) Native MS *m*/z data for MSP treated with 0.5 mM **1** followed with treatment by 0.5 mM PEG_2 -biotin azide (orange).

Extraction of Hen Egg proteins from commercial chicken eggs

6 fresh commercial chicken eggs were broken, and the contents were collected in a Tupperware® container. The egg contents were then stirred thoroughly with a metal spatula. The egg protein solution was then diluted with 50 mL of lysis buffer (50 mL Tris pH 7.4, 2 mM EDTA, 2 mM EGTA, 1% NP-40) and thoroughly stirred prior to transfer to 50 mL conical tubes. After capping the tubes, they were shaken further. Samples were then centrifuged at 4,000 rpm for 20 minutes at 4 ^{o}C for clarification. Samples were then diluted by transferring 10 mL of lysis buffer extract into 40 mL of 0.1 M PBS (pH 6.5). Samples were then centrifuged a second time (4,000 rpm, 20 minutes, 4 ^{o}C). The remaining contents were removed to fresh conical tubes and stored indefinitely at 4 ^{o}C.

*Treatment of Hen Egg protein extract with **1***

234 µL of Hen Egg protein extract (pH 6.5) was treated with 16 µL of 12.6 mM **1** to provide a final treatment of 0.8 mM. The control sample was treated with 16 µL of DMSO in place of **1**. Samples were incubated overnight (~16 hours) void of light. 10 µL of each sample was treated with 2 µL of 5 mM Alexa Fluor azide to provide a final concentration of 1 mM. The click reaction proceeded for 2 hours prior to analysis on SDS-PAGE.

*Test for bioorthogonal capability of BSA modified by **1***

25 µL of 1mM BSA was transferred to two 1.6 mL Eppendorf tubes. To each was added 209 µL of 0.1 M PBS buffer (pH 7). To the control sample, 16 µL of DMSO was added. For the experimental sample, 16 µL of 12.6 mM **1** was added to provide a total volume of 250 µL and a final protein concentration of 100 µM and a final TBD concentration of 0.8 mM (8 equiv). Samples were incubated overnight void of light. 2.5 µL of each sample was then transferred to respective Eppendorf tubes. To this first set was added 7.1 µL of 0.1 M PBS buffer (pH 7), as well as 0.4 µL of 5 mM Alexa Fluor azide to provide a final protein concentration of 25 µM, and a final fluorophore concentration of 0.2 mM (~1:1 equiv **1**: azide). Samples were then incubated for 6 hours void of light at room temperature. In a second set of samples, 2.5 µL of the control and the **1** treated BSA was transferred to Eppendorf tubes containing 7.1 µL of Hen Egg protein extract. These samples were further treated with 0.4 µL of 5 mM Alexa Fluor azide to provide a final protein concentration of 25 µM, and a final fluorophore concentration of 0.2 mM (~1:1 equiv **1**: azide). All samples were then loaded on SDS-PAGE and analyzed.

Absorbance Spectra

TBD-cyclooctyne (1)

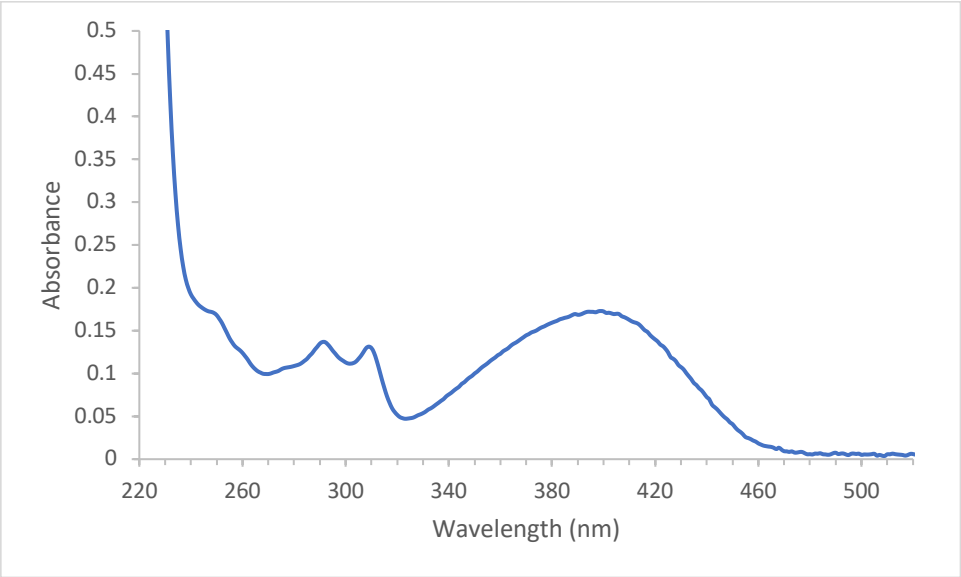


Figure S5. A 6.3 μM stock solution of **1** in DMSO was diluted into 2 mL of MeOH and scanned for absorbance from 200 nm to 550 nm.

References

(1) Reid, D.J.; Keener, J.E.; Wheeler, A.P.; Zambrano, D.E.; Diesing, J.M.; Reinhardt-Szyba, M.; Makarov, A.; Marty, M.T. Engineering Nanodisc Scaffold Proteins for Native Mass Spectrometry. *Anal. Chem.* **2017**, *89* (21), 11189–11192.

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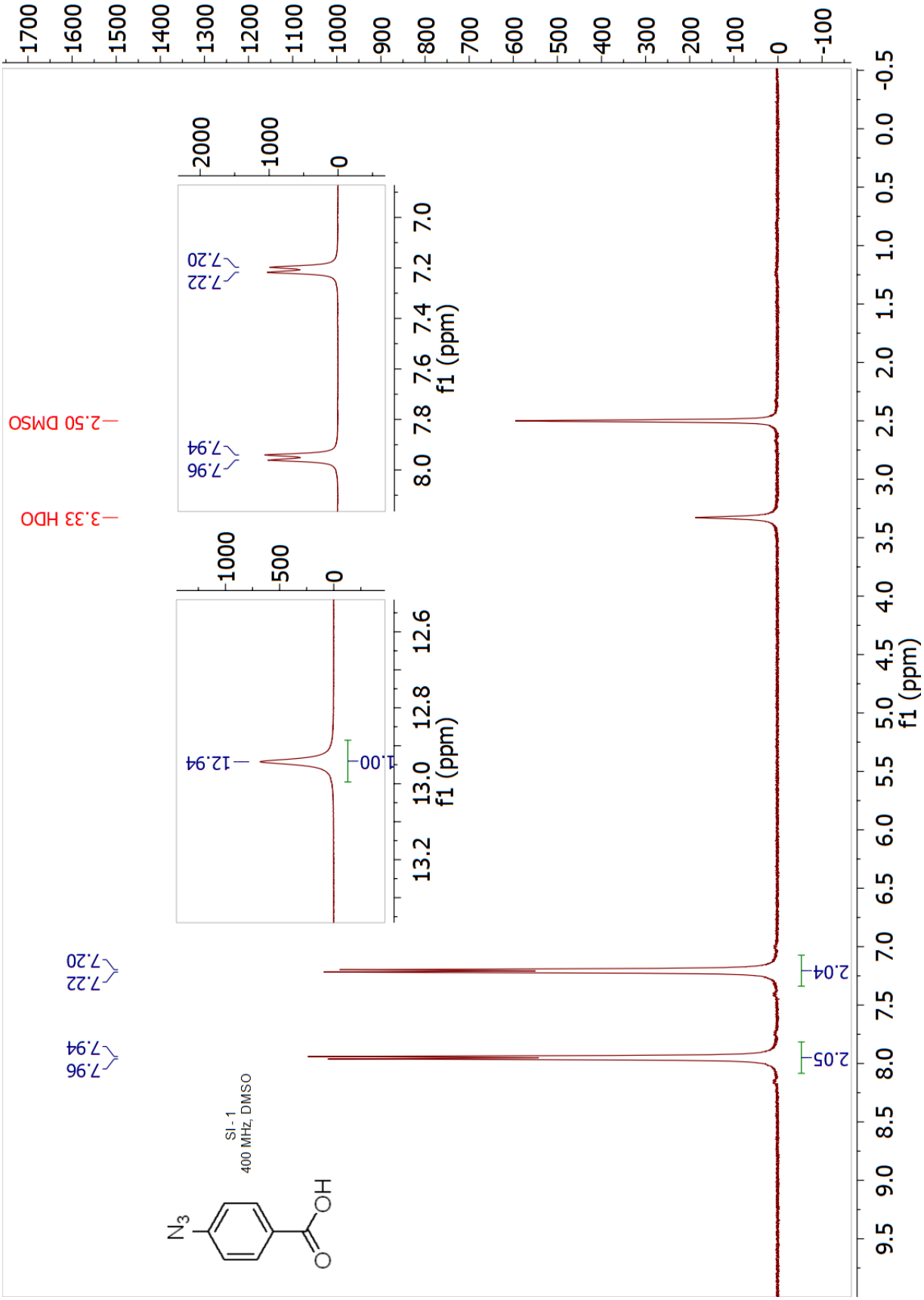
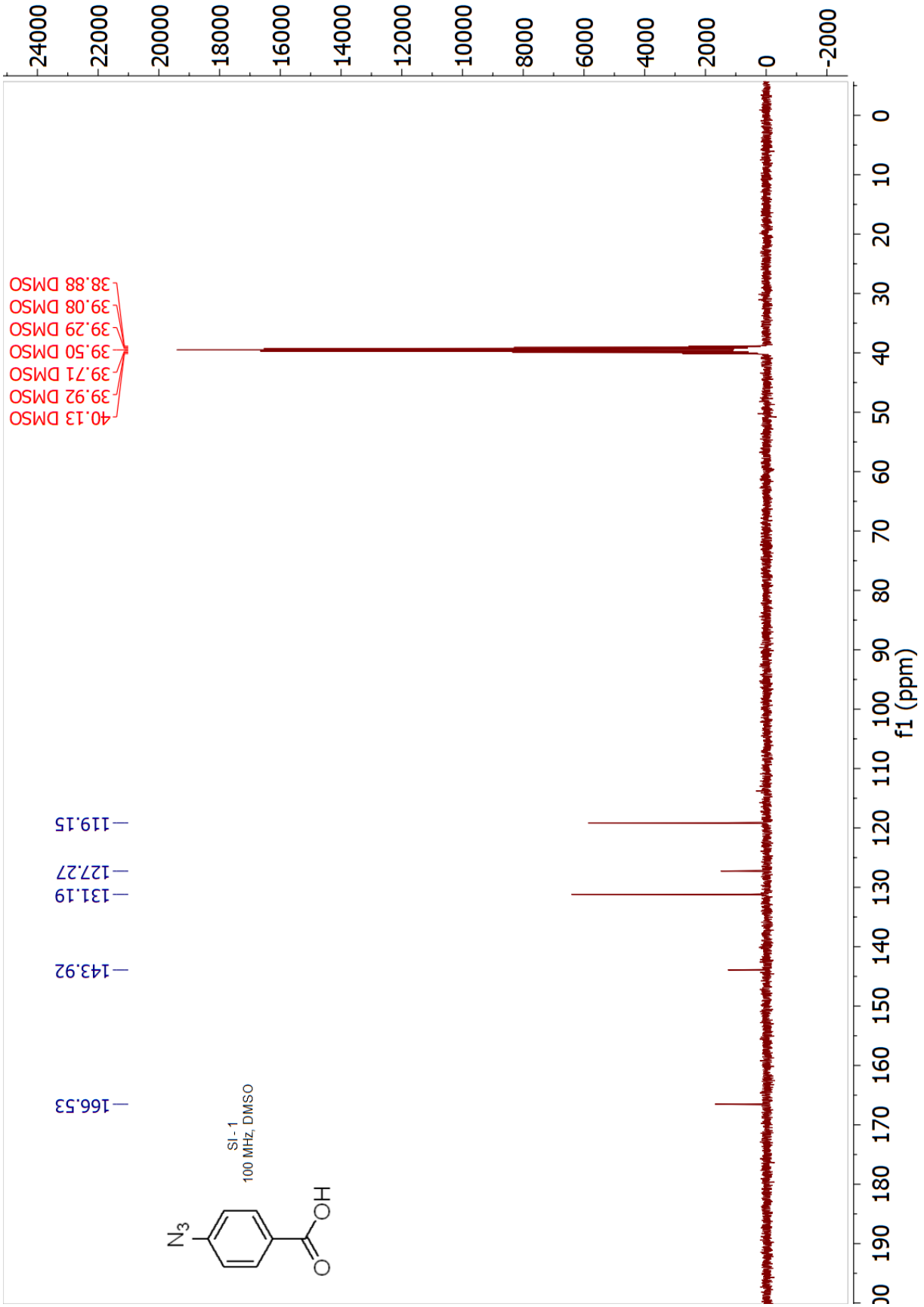
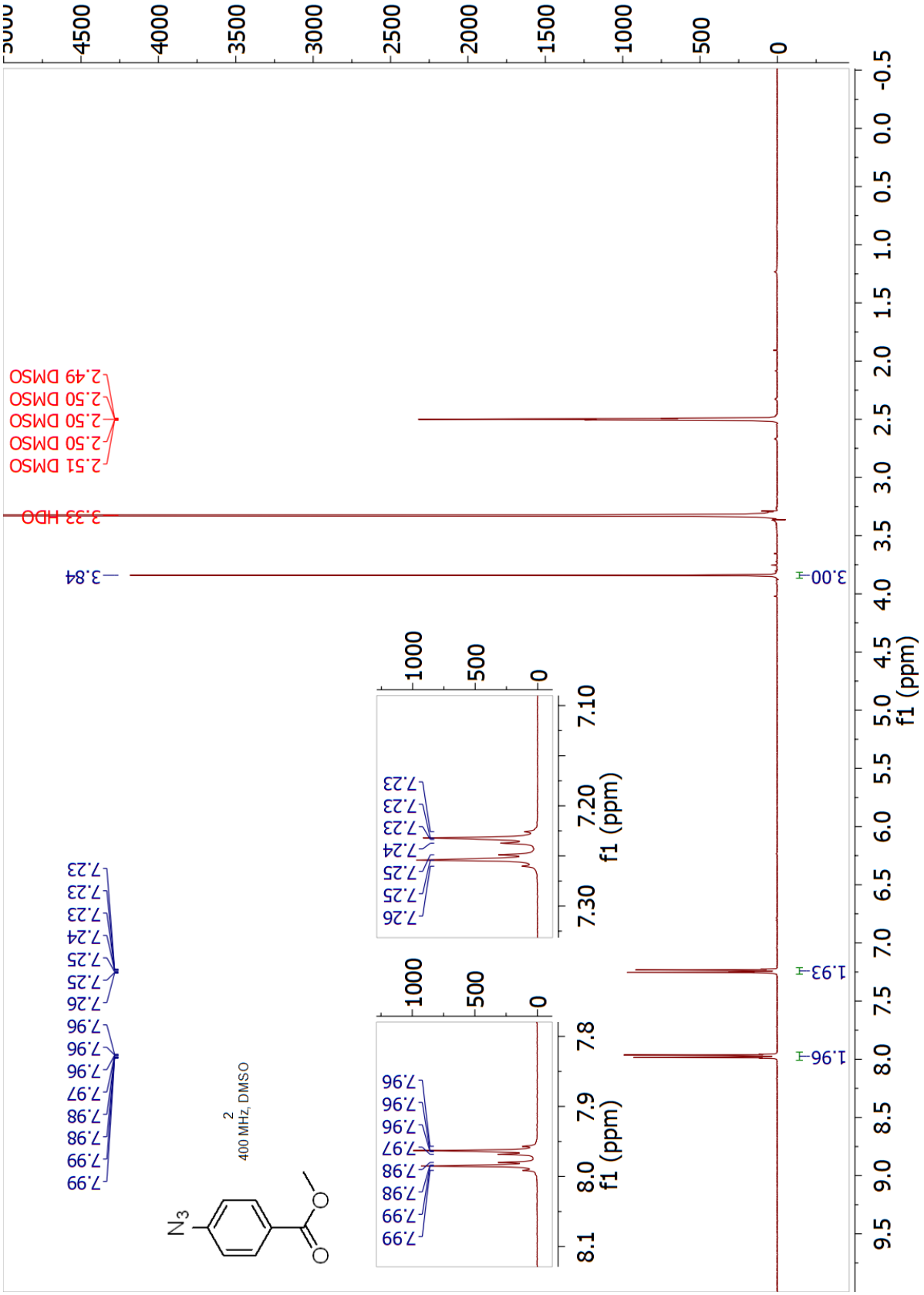
NMR Spectra

NMR spectra were taken on a Bruker AVIII-400 NMR Spectrometer for ^{1}H and ^{13}C NMR and referenced with residual solvent peaks at 7.26 ppm and 77.0 ppm for $CDCl_3$, 2.50 and 39.5 ppm for DMSO-d_6 unless specified. Only the NMR of the final product (**1**) was obtained using Bruker AVIII-500 NMR.


SI-1
400 MHz, DMSO
7.96
7.94
7.22
7.20
2.05
2.04
3.33 HDO
2.50 DMSO
12.94
1.00
f1 (ppm)


SI - 1
100 MHz, DMSO
166.53
143.92
131.19
127.27
119.15
40.13 DMSO
39.92 DMSO
39.71 DMSO
39.50 DMSO
39.29 DMSO
39.08 DMSO
38.88 DMSO
f1 (ppm)


400 MHz, DMSO
f1 (ppm)
3.84
3.33 HDO
2.51 DMSO
2.50 DMSO
2.50 DMSO
2.50 DMSO
2.49 DMSO
7.99
7.99
7.98
7.98
7.97
7.96
7.96
7.96
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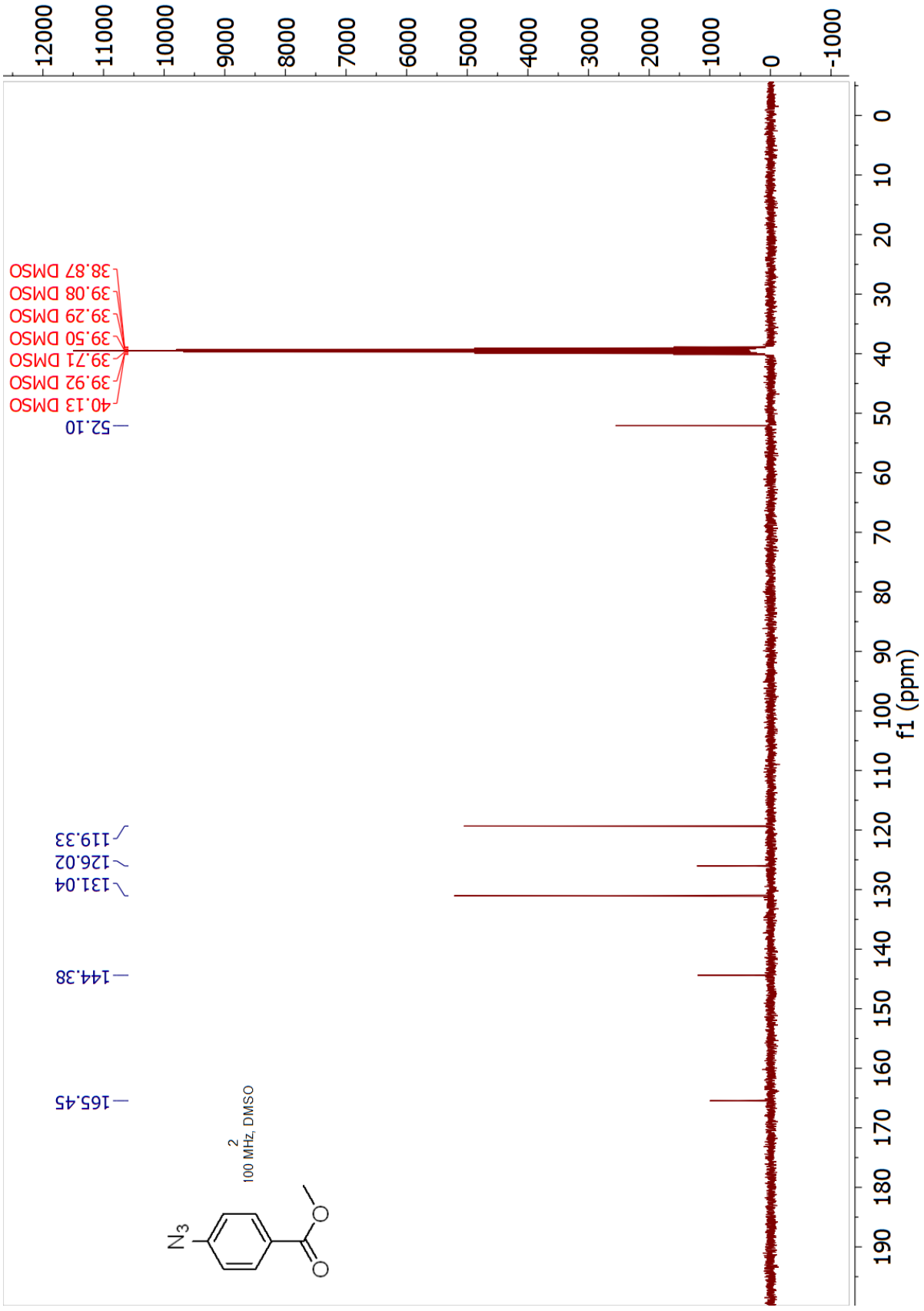
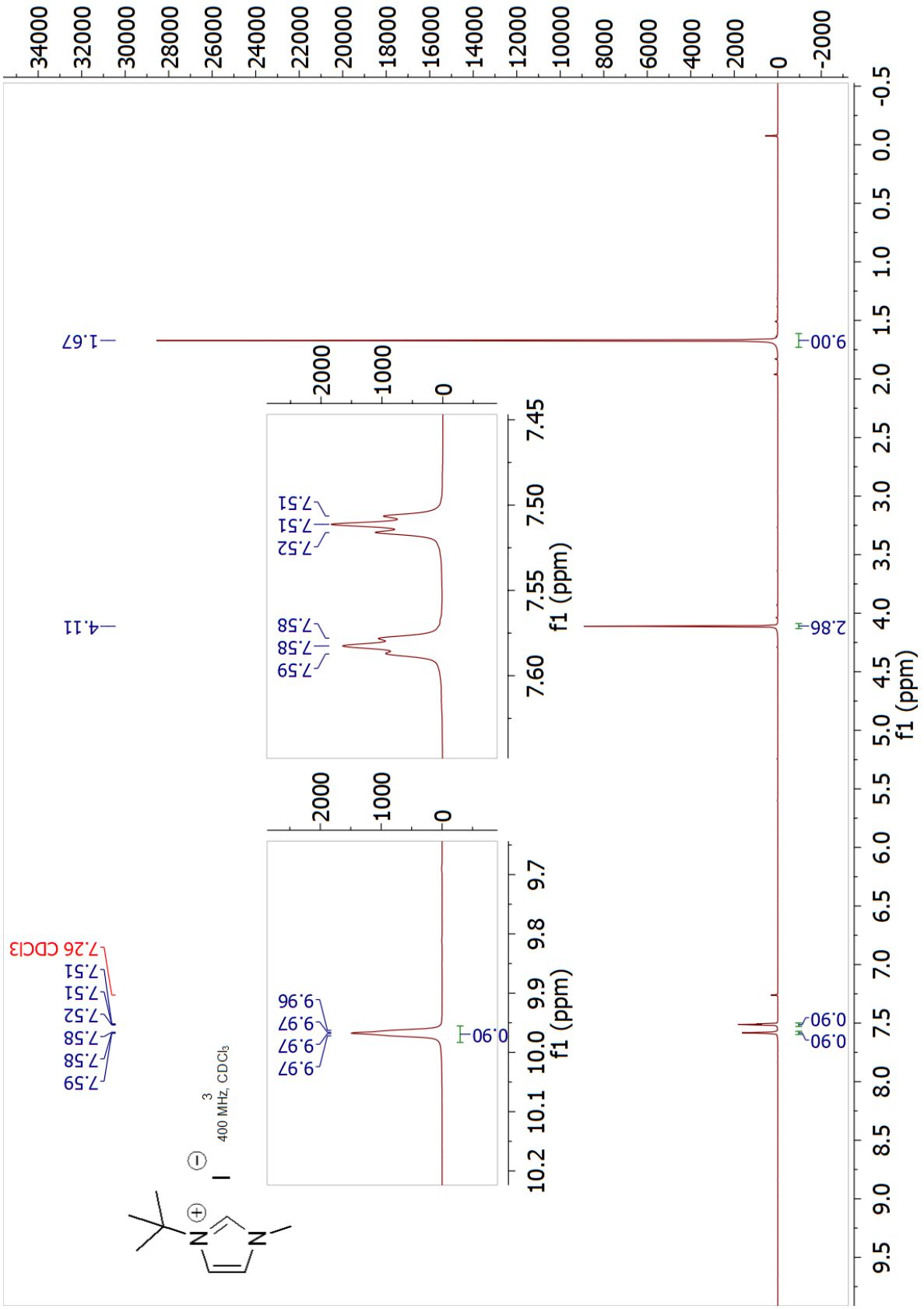
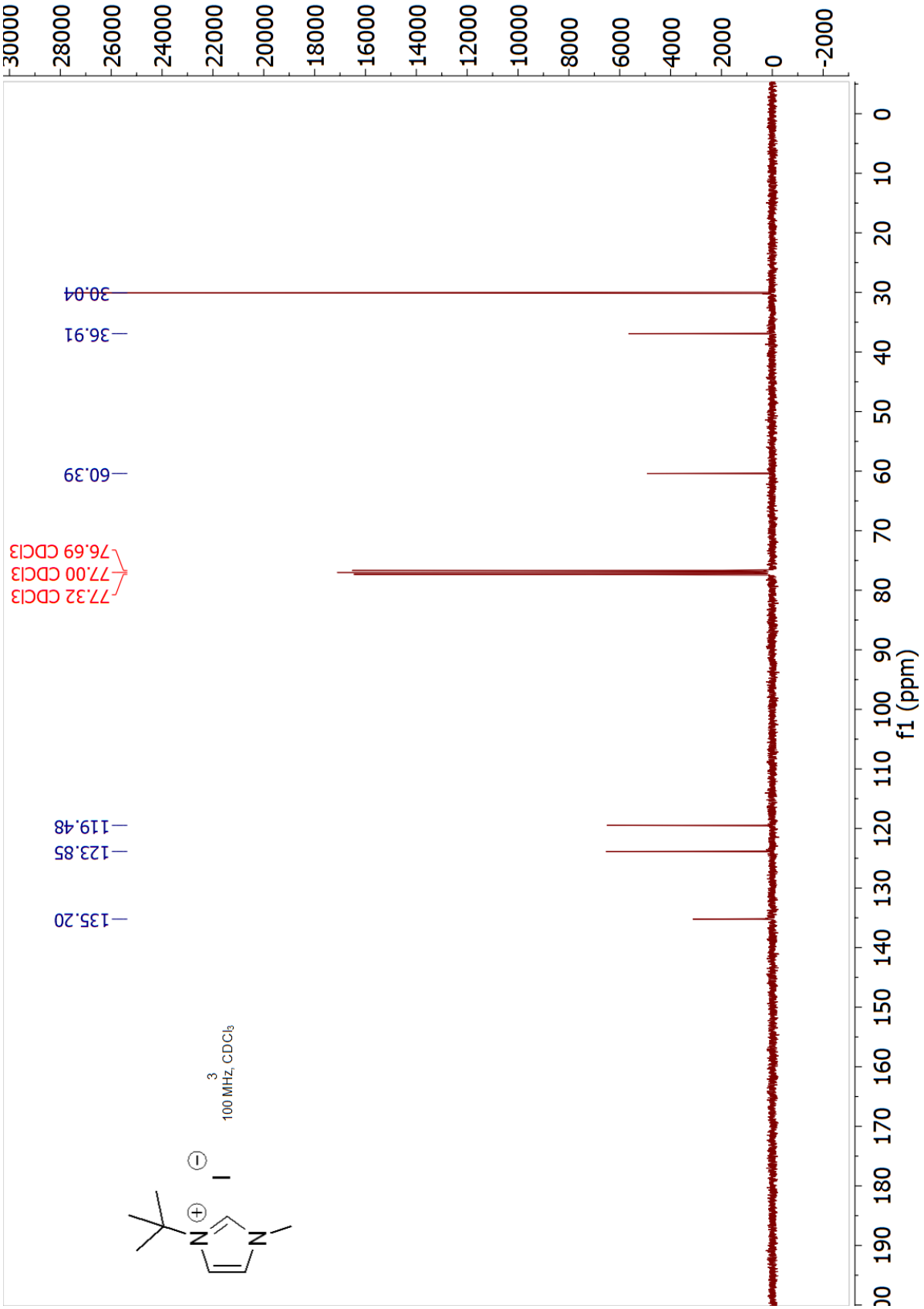
7.23
7.23
7.23
3.00
1.93
1.96


12000
11000
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
-1000
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
190
f1 (ppm)
38.87 DMSO
39.08 DMSO
39.29 DMSO
39.50 DMSO
39.71 DMSO
39.92 DMSO
40.13 DMSO
52.10
119.33
126.02
131.04
144.38
165.45
2
100 MHz, DMSO
N3
O
O


400 MHz, CDCl3
7.26 CDCl3
f1 (ppm)


30000
28000
26000
24000
22000
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
-2000
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
190
200
f1 (ppm)
30.04
36.91
60.39
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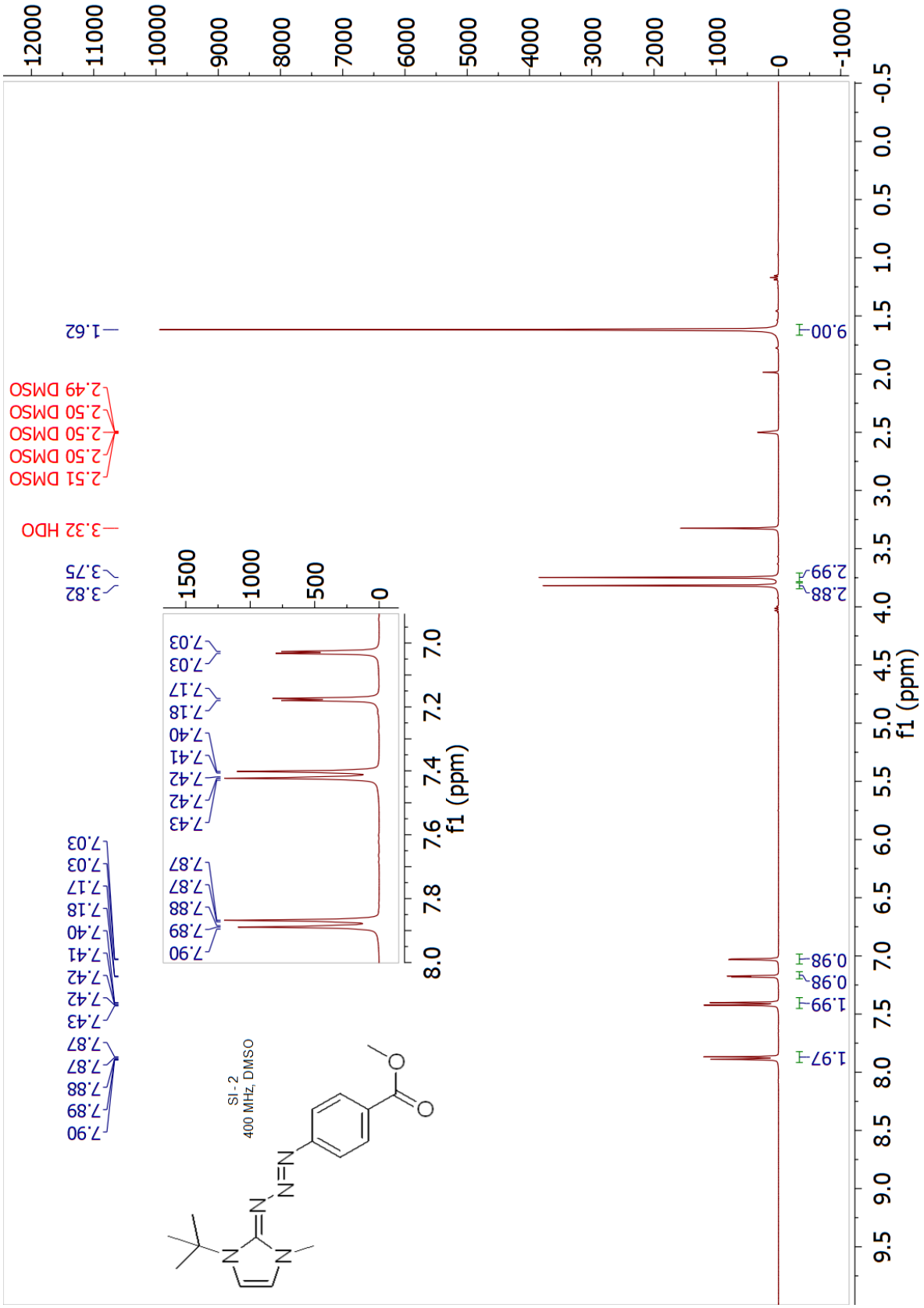
77.00 CDCl3
77.32 CDCl3
119.48
123.85
135.20
100 MHz, CDCl3
I
N
N


SI - 2
400 MHz, DMSO
f1 (ppm)
9.00
2.88
2.99
1.97
1.99
0.98
0.98
1.62
3.32 HDO
2.51 DMSO
2.50 DMSO
2.50 DMSO
2.50 DMSO
2.49 DMSO
3.75
3.82
7.90
7.89
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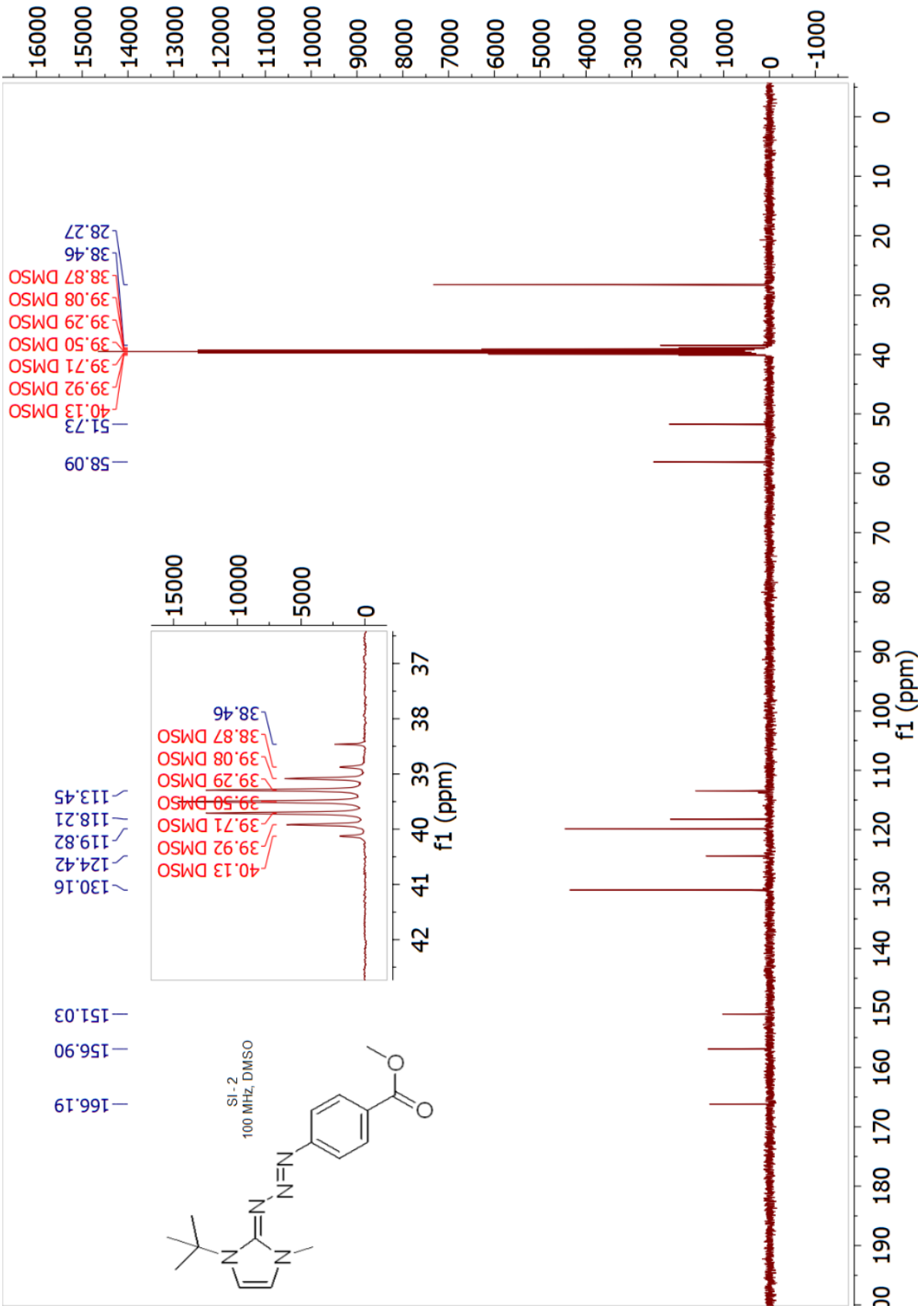
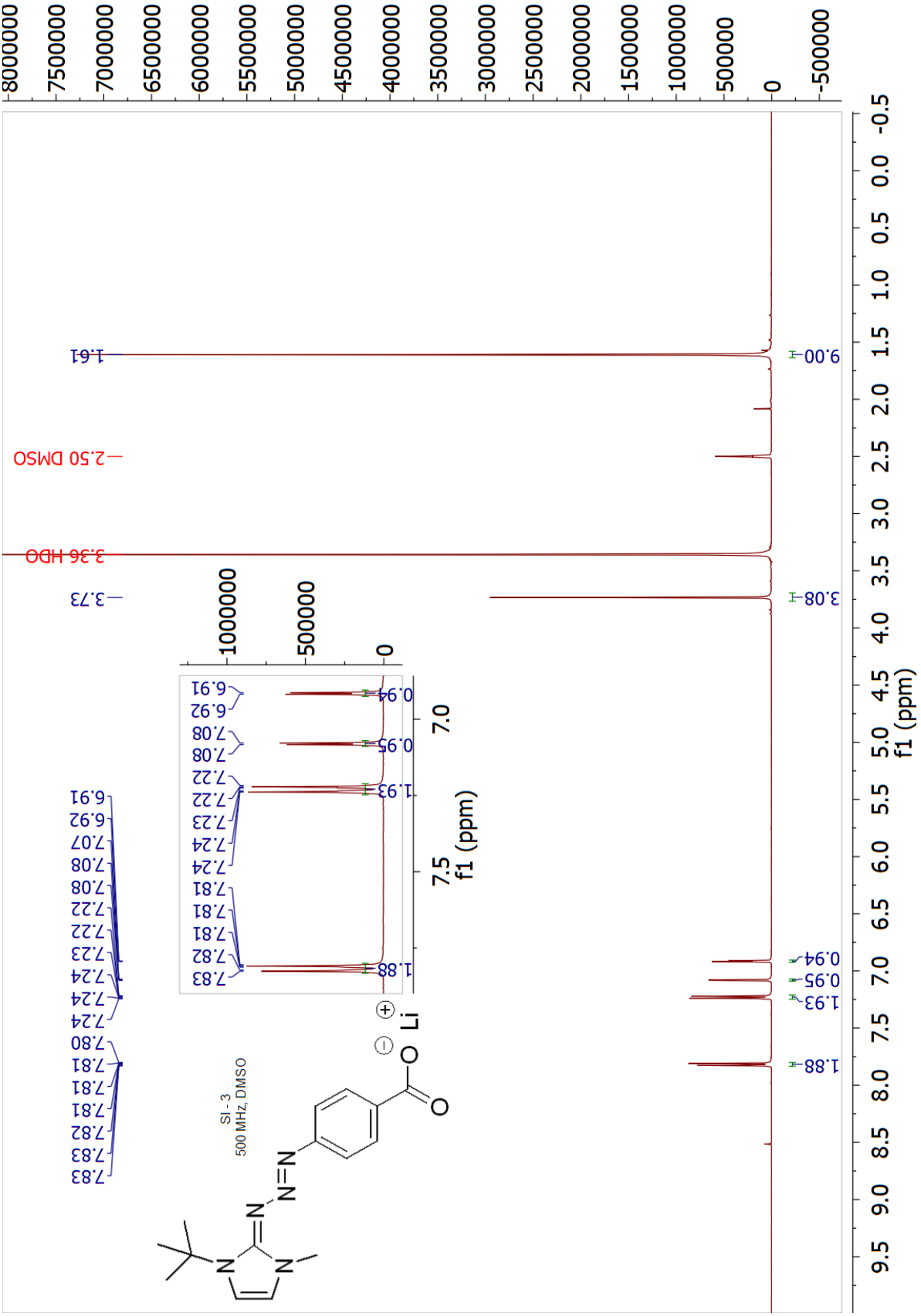
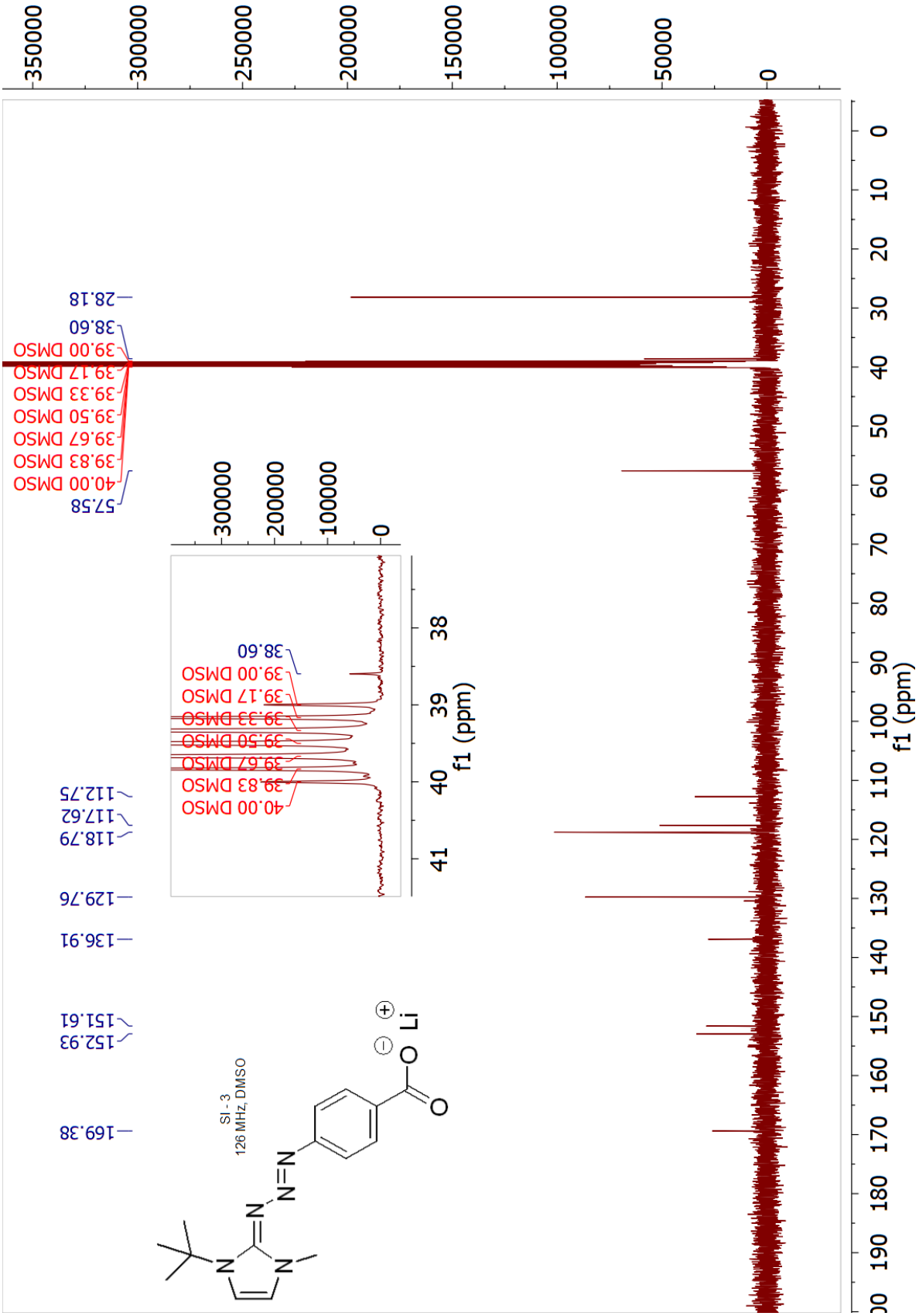
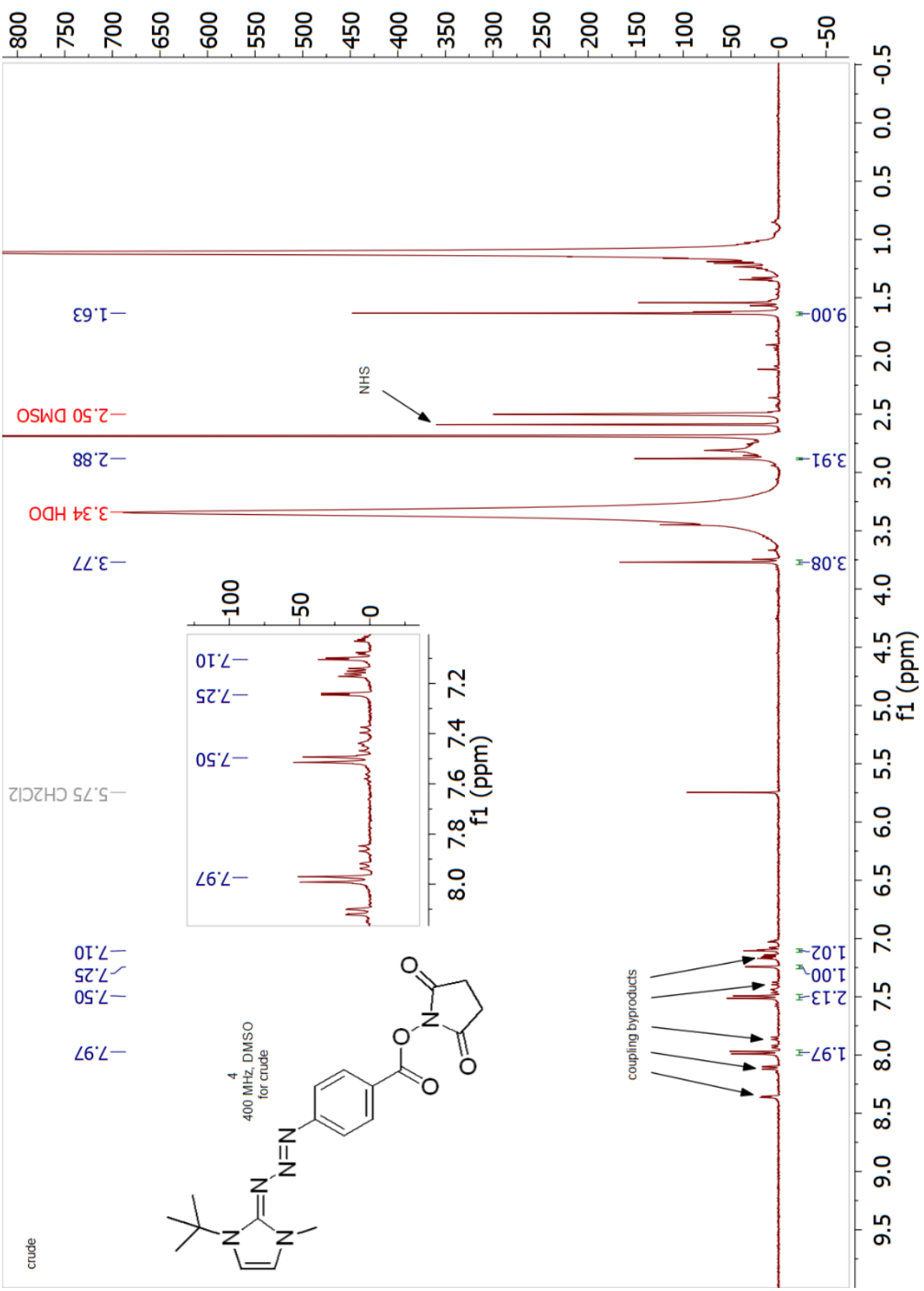
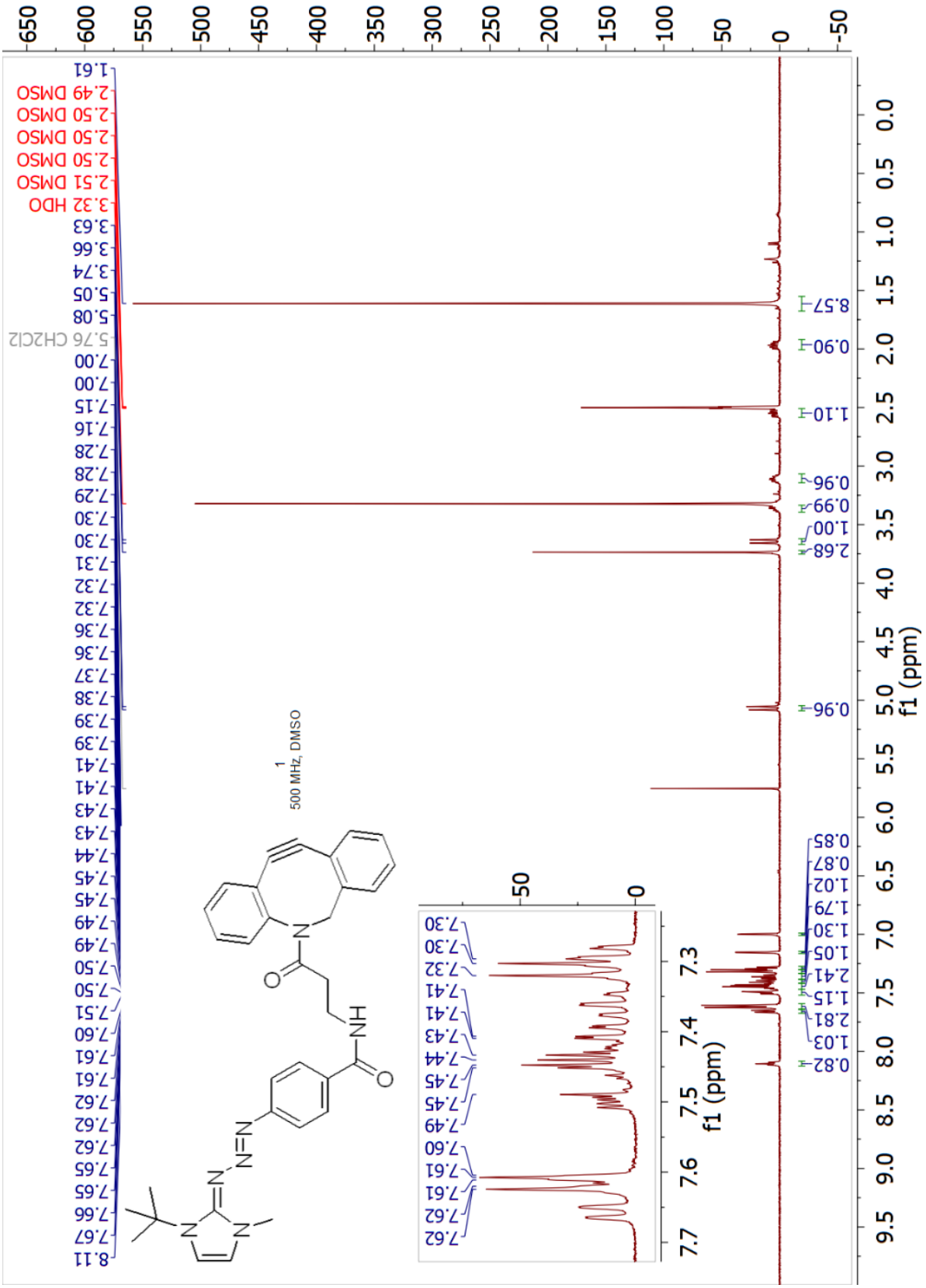
7.41
7.40
7.18
7.17
7.03
7.03

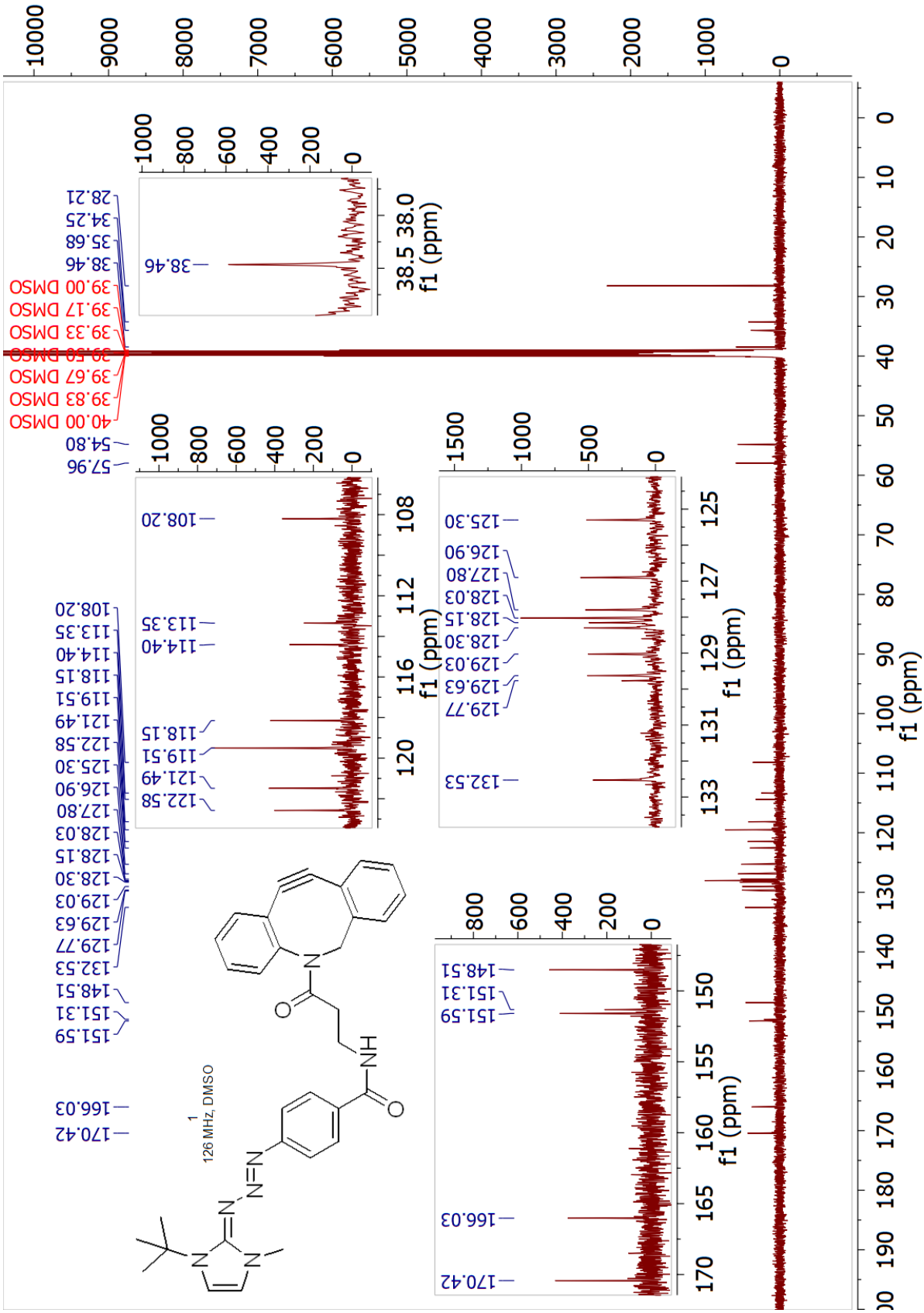

SI - 2
100 MHz, DMSO
f1 (ppm)
166.19
156.90
151.03
130.16
124.42
119.82
118.21
113.45
58.09
51.73
40.13 DMSO
39.92 DMSO
39.71 DMSO
39.50 DMSO
39.29 DMSO
39.08 DMSO
38.87 DMSO
38.46
28.27

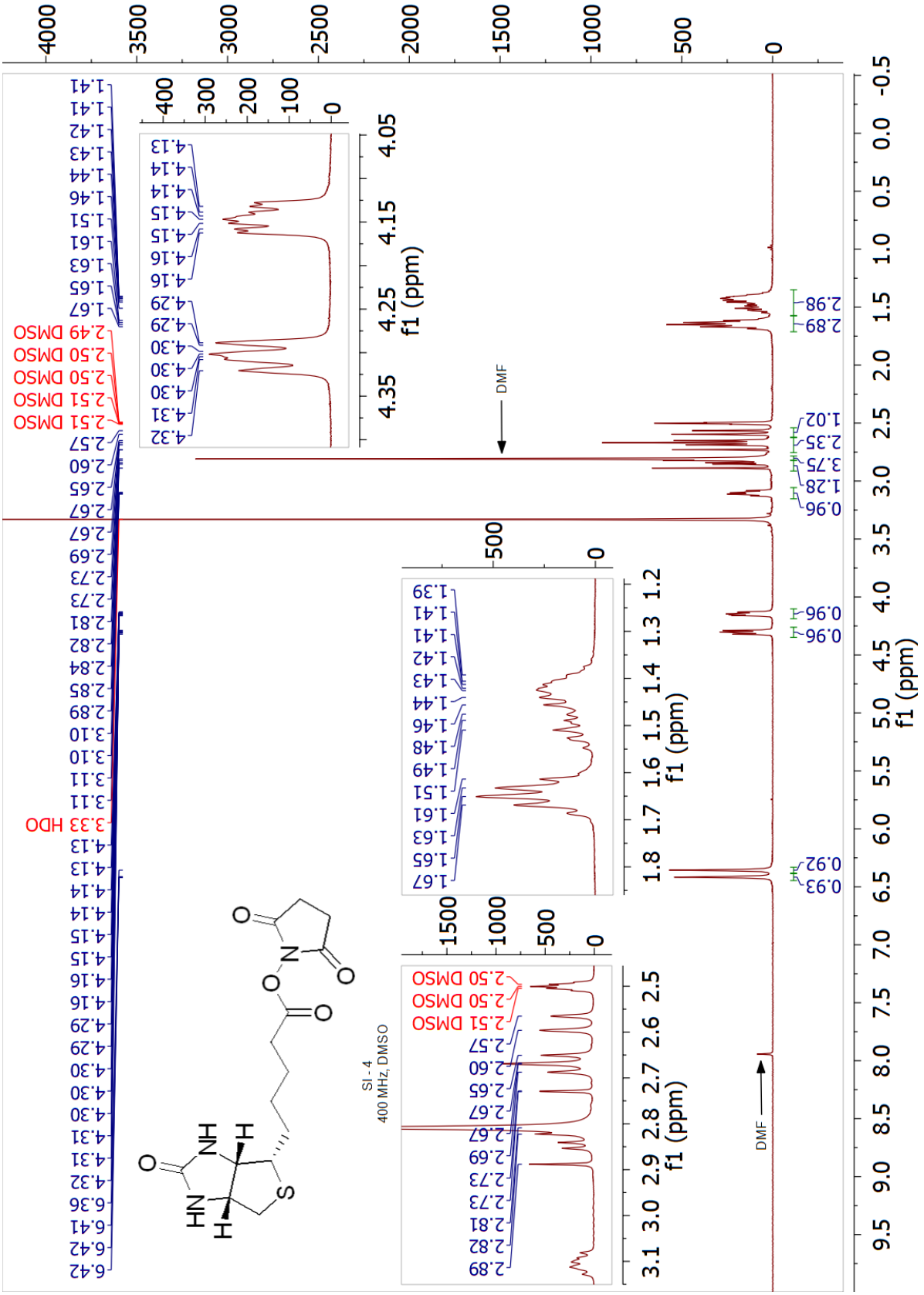

1.61
2.50 DMSO
3.36 HDO
3.73
7.83
7.82
7.81
7.81
7.81
7.80
7.24
7.24
7.24
7.23
7.22
7.22
7.08
7.07
6.92
6.91
9.00
3.08
1.93
0.95
0.94
1.88
SI - 3
500 MHz, DMSO
Li
O
O
N
N
N
N
f1 (ppm)


SI - 3
126 MHz, DMSO
Li
O
O
N
N
N
N
f1 (ppm)
28.18
38.60
39.00 DMSO
39.17 DMSO
39.33 DMSO
39.50 DMSO
39.67 DMSO
39.83 DMSO
40.00 DMSO
57.58
112.75
117.62
118.79
129.76
136.91
151.61
152.93
169.38


crude
4
400 MHz, DMSO
for crude
NHS
coupling byproducts
f1 (ppm)


1
500 MHz, DMSO
f1 (ppm)


126 MHz, DMSO
f1 (ppm)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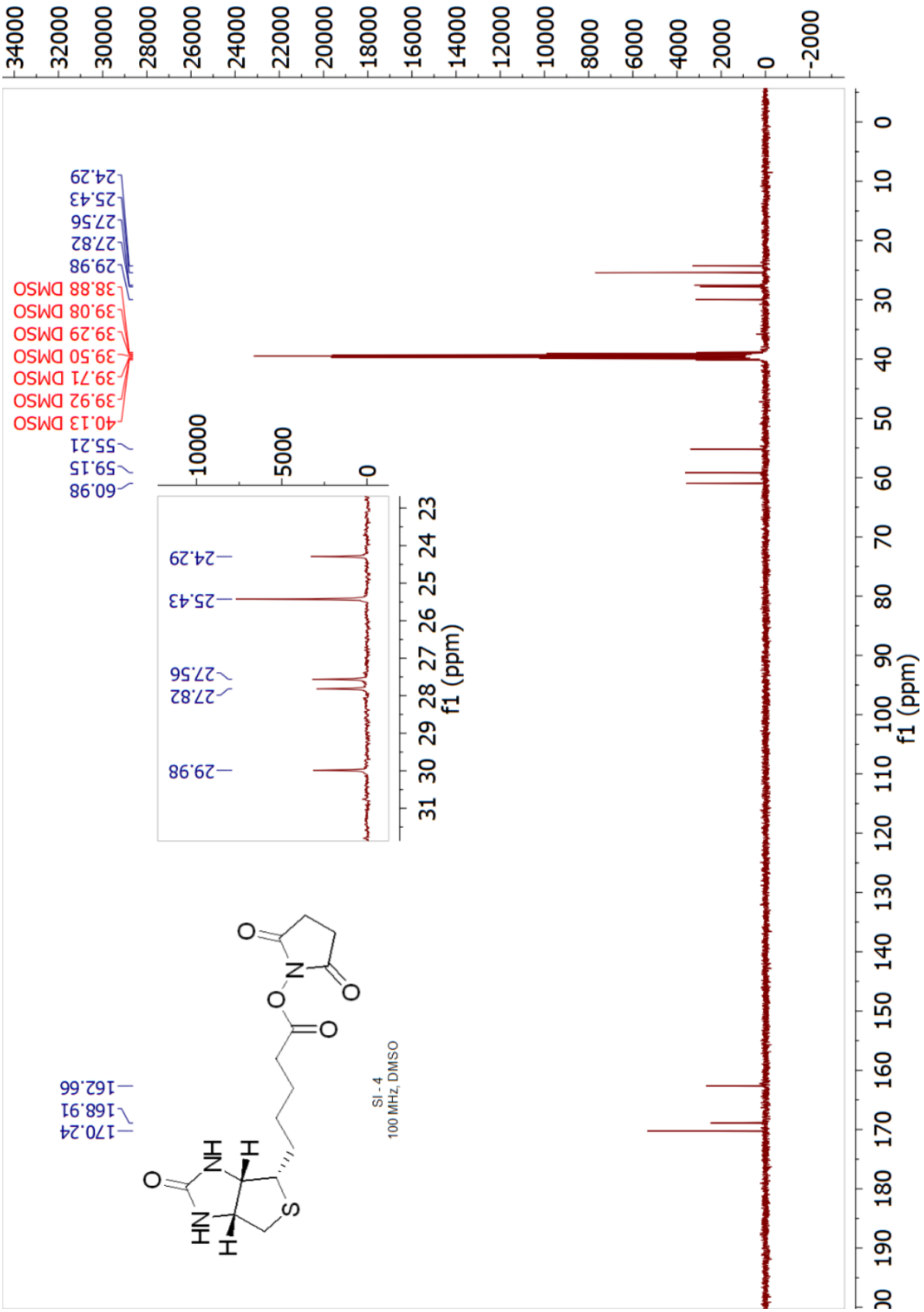
400 MHz, DMSO
DMF
DMF
f1 (ppm)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100 MHz, DMSO
f1 (ppm)
170.24
168.91
162.66
60.98
59.15
55.21
40.13 DMSO
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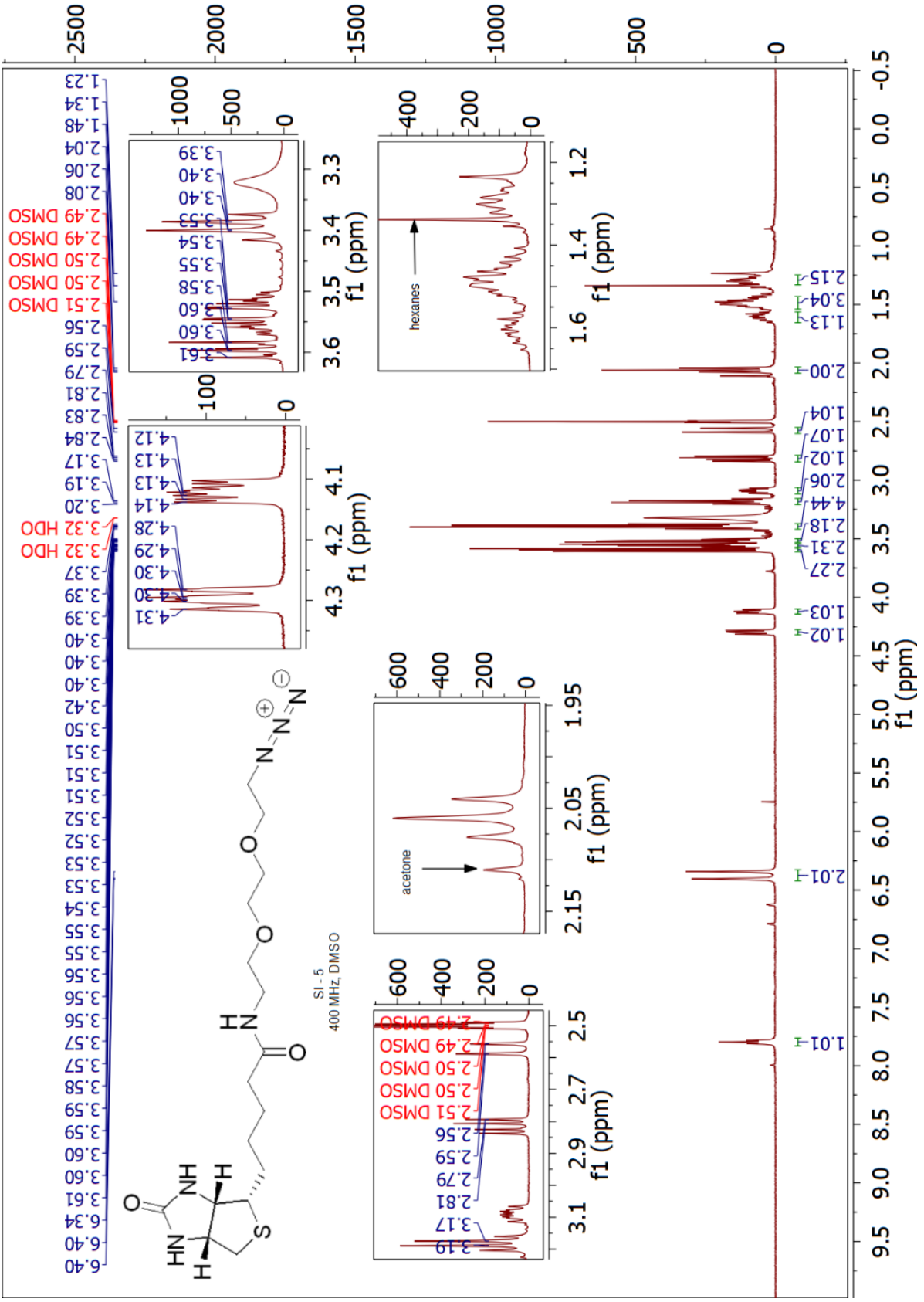
29.98
27.82
27.56
25.43
24.29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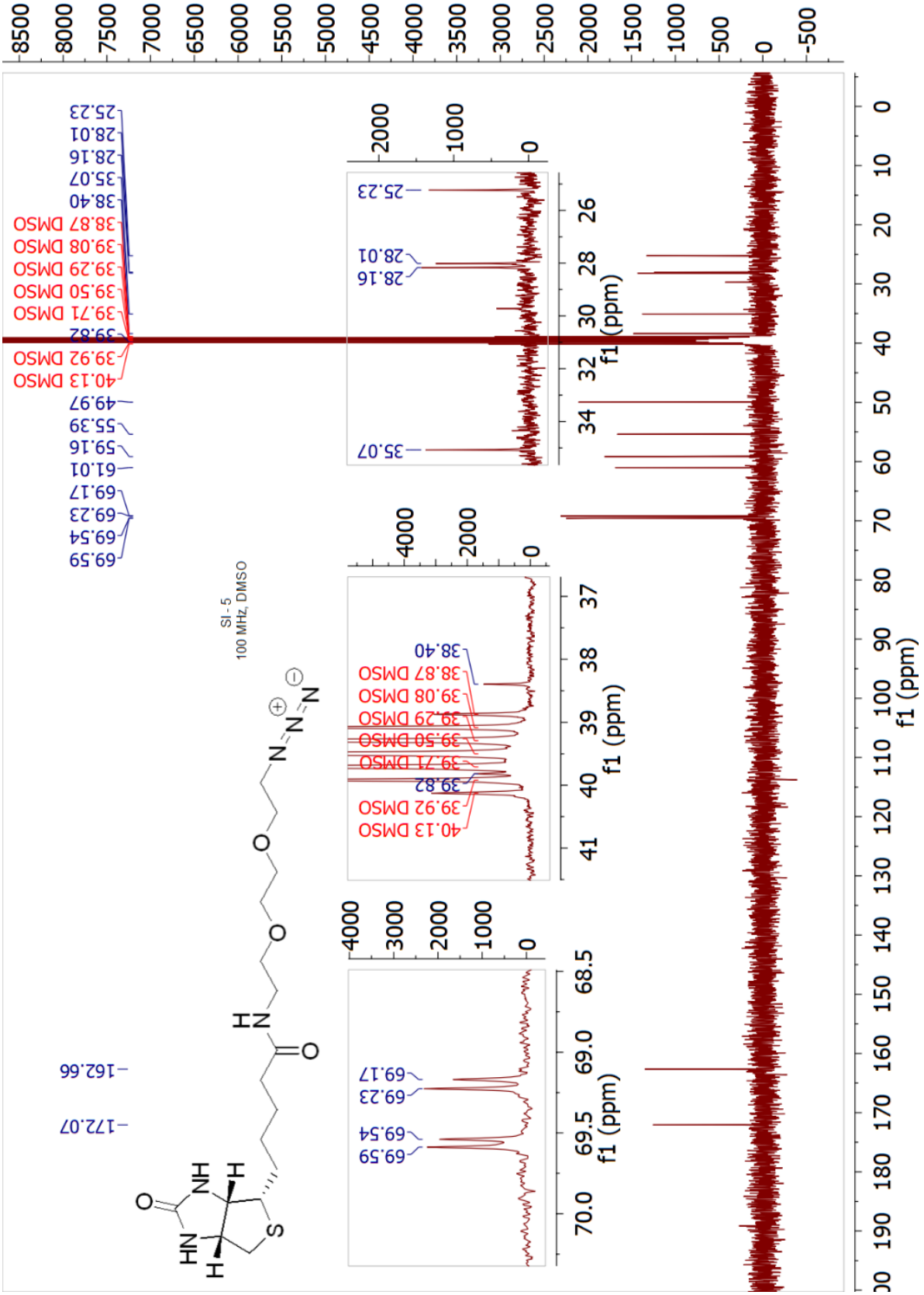
400 MHz, DMSO
f1 (ppm)
acetone
hexanes
2.49 DMSO
2.50 DMSO
2.51 DMSO
3.32 HDO


SI - 5
100 MHz, DMSO
f1 (ppm)