Supplementary Information

Methods for converting cysteine to dehydroalanine on peptides and proteins

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General Considerations

Melting points (m.p.) were recorded on a Leica Galen III hot stage microscope equipped with a Testo 720 thermocouple probe. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Bruker DPX200 (200 MHz), a Bruker AV400 (400 MHz), or a Bruker AVII500 (500 MHz) spectrometer, as indicated. Carbon nuclear magnetic resonance (^{13}C NMR) spectra were recorded on a Bruker DPX200 (50 MHz), a Bruker AV400 (100 MHz) or on a Bruker AVII500 (125 MHz) spectrometer, as indicated. NMR Spectra were assigned using COSY, HMQC, and DEPT 135. All chemical shifts are quoted on the δ scale in ppm using residual solvent as the internal standard (^{1}H NMR: $CDCl_3$ = 7.26, CD_3OD = 3.31; DMSO-d_6 = 2.50; CD_3CN = 1.94 and ^{13}C NMR: $CDCl_3$ = 77.0; CD_3OD = 49.0; DMSO-d_6 = 39.5; CD_3CN = 1.94). Coupling constants (*J*) are reported in Hz with the following splitting abbreviations: s = singlet, d = doublet, t = triplet, q = quartet, quin = quintet, and a = apparent.

Infrared (IR) spectra were recorded on a Bruker Tensor 27 Fourier Transform spectrophotometer using thin films on NaCl plates for liquids and oils and KBr discs for solids and crystals. Absorption maxima (υ_{max}) are reported in wavenumbers (cm^{-1}).

Low resolution mass spectra (LRMS) were recorded on a Waters Micromass LCT Premier TOF spectrometer using electrospray ionization (ESI) and high resolution mass spectra (HRMS) were recorded on a Bruker MicroTOF ESI mass spectrometer. Nominal and exact m/z values are reported in Daltons. Other methods of ionization (EI, FI, and FAB) are used where indicated and were recorded by the University of Oxford Mass Spectrometry Service in the Department of Chemistry.

Optical rotations were measured on a Perkin-Elmer 241 polarimeter with a path length of 1.0 dm and are reported with implied units of 10^{-1} deg cm^2 g^{-1}. Concentrations (c) are given in g/100 ml.

Thin layer chromatography (TLC) was carried out using Merck aluminium backed sheets coated with $60F_{254}$ silica gel. Visualization of the silica plates was achieved using a UV lamp (λ_{max} = 254 nm), and/or ammonium molybdate (5% in 2M H_2SO_4), and/or potassium permanganate (5% $KMnO_4$ in 1M NaOH with 5% potassium carbonate). Flash column chromatography was carried out using BDH PROLAB® 40-63 mm silica gel (VWR). Mobile phases are reported in % volume of more polar solvent in less polar solvent for binary systems (e.g. 20% EtOAc in petrol = 1:4

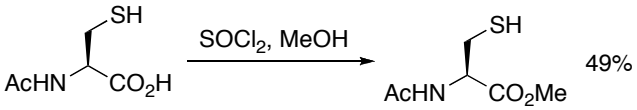
ethyl acetate:petrol) and in relative composition for ternary systems (e.g. 1:2:4 H_2O:^{i}PrOH:EtOAc)

Anhydrous solvents were purchased from Fluka or Acros with the exception of dichloromethane and THF, which were dried in an alumina column under nitrogen. Triethylamine was stored over NaOH pellets. All other solvents were used as supplied (Analytical or HPLC grade), without prior purification. Deionized water was used for chemical reactions and Milli-Q purified water for protein manipulations. Protein concentrations were determined by BCA (bicinchoninic acid) assay (Pierce) and/or OD_{280}. Reagents were purchased from Sigma-Aldrich and used as supplied, unless otherwise indicated. 'Petrol' refers to the fraction of light petroleum ether boiling in the range 40-60 ºC. All reactions using anhydrous conditions were performed using flame-dried apparatus under an atmosphere of argon or nitrogen. Brine refers to a saturated solution of sodium chloride. Anhydrous magnesium sulfate ($MgSO_4$) or sodium sulfate (Na_2SO_4) were used as drying agents after reaction workup, as indicated.

DOWEX-50WX8 (H^+ form) was conditioned as follows: 100 g of the commercial resin was placed in a 500 mL sintered filter funnel and allowed to swell with 200 mL of acetone for 5 minutes. The solvent was removed by suction and the resin was washed successively with 800 mL of acetone, 500 mL methanol, 500 mL 5M HCl, and then 1 L of water or until the pH of filtrate was ~ 7, as indicated by pH paper. The resin was partially dried on the filter and then stored and used as needed.

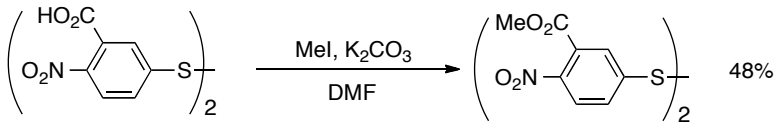
Reductive Route to Dha Using HMPT

AcCysOMe



N-Acetyl cysteine (5.00 g, 30.6 mmol) was added to a flame dried 250 mL round bottom flask under argon and dissolved in MeOH (50 mL). The stirred solution was cooled to 0 ºC and thionyl chloride (2.46 mL, 33.7 mmol) was added slowly. The reaction was stirred for 10 minutes at 0 ºC and then at room temperature for 1.5 hours. The solvent was then removed under reduced pressure. The resulting residue was redissolved in EtOAc (250 mL) and washed successively with H_2O (200 mL) and brine (200 mL). The organic layer was then dried ($MgSO_4$), filtered, and concentrated under reduced pressure to give a thick residue that crystallized on standing (2.64 g, 49%). This material was used without further purification. Spectroscopic data was consistent with that previously reported.[1] m.p. = 51-52 ºC. $[\alpha]^{20}_D$ = +96.0 (c = 1.3, $CHCl_3$). IR (υ_{max}, KBr): 3281, 1743, 1656, 1541, 1437, 1374, 1217. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.37 (1H, t, *J* = 9.0, SH), 2.03 (3H, s, NHAc), 2.93-2.97 (2H, m, C<u>H</u>$_2$SH), 3.74 (3H, s, $CO_2C\underline{H}_3$), 4.82-4.86 (1H, m, Hα), 6.66 (1H, d, *J* = 6.3, NH). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 23.1 (NHAc), 26.8 (<u>C</u>H_2SH), 52.8, 53.6 ($CO_2\underline{C}H_3$, Cα), 170.1, 170.8 (C=O). LRMS m/z (ESI$^-$): 176 [M-H]$^-$.

Methyl ester of Ellman's reagent (4)



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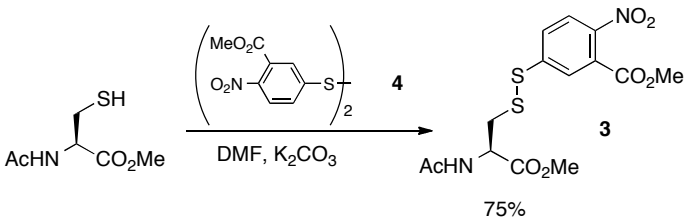
Ellman's reagent (8.00 g, 20.2 mmol) was added to a 100 mL round bottom flask and dissolved in DMF (40 mL). The stirred solution was cooled to 0 ºC and K_2CO_3 (14.0 g, 101 mmol) was added. Methyl iodide (3.77 mL, 60.6 mmol) was added dropwise and the reaction stirred (open air) from 0 ºC to room temperature over 2 hours. The reaction was diluted with EtOAc (300 mL) and then washed successively with $NaHCO_3$ (sat. aq., 2 × 100 mL), H_2O (2 × 100 mL), and brine (2 × 100 mL). The organic layer was dried ($MgSO_4$), filtered, and concentrated to a thick yellow oil. The oil was dissolved in 25 mL of hot toluene (~50 ºC) and petrol was added dropwise to the stirred solution until turbid. Additional hot toluene was added until the solution was clear. The solution was left to crystallize. The resulting yellow prisms were isolated by filtration and dried under vacuum (4.15 g, 48%). m.p. = 100-102 ºC. IR (υ_{max}, KBr): 2850, 2361, 1738, 1568, 1527, 1435,

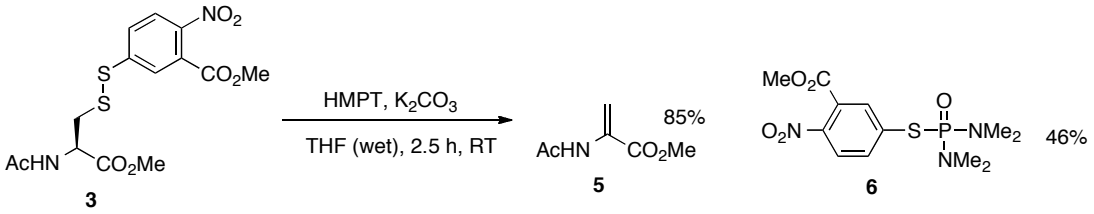
1341, 1281, 1134, 1101, 1060. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 3.92 (6H, s, $CO_2C\underline{H}_3$), 7.68 (2H, dd, *J* = 8.6, 2.0, ArH), 7.75 (2H, d, *J* = 2.0, ArH), 7.92 (2H, d, *J* = 8.6, ArH). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 53.5 ($CO_2\underline{C}H_3$), 125.1, 126.7, 128.3 (CH_{Ar}), 128.9, 142.4, 146.4 (4^{o}_{Ar}), 165.1 (C=O). HRMS m/z (ESI^+): Found 446.9925 $[M+Na]^+$; $C_{16}H_{12}N_2O_8S_2Na$ requires 446.9927. Analysis for $C_{16}H_{12}N_2O_8S_2$ requires: C, 45.28%; H, 2.85%; N, 6.60%; Found: C, 45.40%; H, 3.04%; N, 6.60%.

Disulfide 3

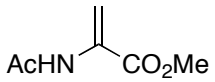


Ellman's methyl ester **4** (2.50 g, 5.89 mmol) was added to a 100 mL round bottom flask along with K_2CO_3 (1.63 g, 11.76 mmol) and DMF (30 mL). To the stirred mixture was added a solution of AcCysOMe (695 mg, 3.92 mmol) in DMF (10 mL) over a 5 minute period. The reaction was stirred for 5 minutes at room temperature (open air) after which time TLC (EtOAc) revealed the desired product (R_f = 0.5) and the thiol from reduced **4** (R_f = 0.8). The reaction was diluted with EtOAc (250 mL) and then washed successively with $NaHCO_3$ (sat. aq. 2 × 100 mL) and brine (2 × 100 mL). The organic layer was then dried ($MgSO_4$), filtered, and concentrated under reduced pressure. The resulting residue was purified by column chromatography (80% EtOAc in petrol) to give **3** as thick yellow oil which crystallized on slow cooling in hot EtOAc. The resulting yellow crystals were isolated by filtration and dried under vacuum (1.14 g, 75%). m.p. = 82-84 ºC. $[\alpha]^{20}_{D}$ = +87.6 (c = 1.2, $CHCl_3$). IR (υ_{max}, KBr): 2980, 1740, 1659, 1525, 1341, 1280. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.97 (3H, s, NHAc), 3.11-3.27 (2H, ABX system, *J* = 14.5, 6.4, 5.2, $C\underline{H}_2S$), 3.68 (3H, s), 3.87 (3H, s) (2 × $CO_2C\underline{H}_3$), 4.77-4.81 (1H, m, Hα), 6.77 (1H, d, *J* = 7.6, NH), 7.65 (1H, dd, *J* = 8.7, 2.2, ArH), 7.71 (1H, d, *J* = 2.2, ArH), 7.89 (1H, d, *J* = 8.7, ArH). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 22.7 (NHAc), 40.4 (CH_2S), 51.4 (Cα), 52.6, 53.3 (2 × $CO_2\underline{C}H_3$), 124.6, 126.0, 127.9 (CH_{Ar}), 128.6, 144.8, 145.3 (4º Ar), 165.5, 170.1, 170.3 (3 × C=O). HRMS m/z (ESI^+): Found 411.0292 $[M+Na]^+$; $C_{14}H_{16}N_2O_7S_2Na$ requires 411.0291.

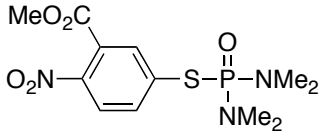
Reaction of 3 with HMPT



Disulfide **3** (32 mg, 0.082 mmol) was added to a 25 mL round bottom flask and dissolved in THF (2 mL, HPLC grade but not anhydrous). K_2CO_3 (57 mg, 0.41 mmol) was added to the stirred solution, followed by HMPT (30 μL, 0.16 mmol). Upon addition of HMPT, the color of the reaction turned red. TLC analysis (EtOAc) after HMPT addition revealed complete consumption of disulfide. The reaction flask was stoppered and the reaction was stirred at room temperature for 2.5 hours. After this time, TLC (EtOAc) indicated the formation of two major products (R_f = 0.7 and R_f = 0.4). The solvent was removed under reduced pressure and the resulting residue was purified by column chromatography (85% EtOAc in petrol). AcDhaOMe (**5**) (10 mg, 85%) and phosphoramide **6** (13 mg, 46% based on disulfide) were isolated.

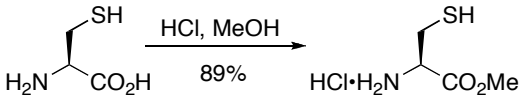


(**5**) Spectroscopic data is consistent with that previously reported.[2] Clear crystals. m.p. = 40-41 ºC. IR (υ_{max}, film): 1730, 1677, 1636, 1519, 1440, 1372, 1235, 1203, 1171. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 2.14 (3H, s, NHAc), 3.85 (3H, s, CO_2CH_3), 5.89 (1H, d, J = 1.6, C=C<u>H</u>H), 6.60 (1H, app. s, C=CH<u>H</u>), 7.73 (1H, br. s, NH). ^{13}C NMR (125 MHz, $CDCl_3$): δ = 24.7 (NHAc), 53.0 (CO_2CH_3), 108.7 (C=<u>C</u>H_2), 130.8 (<u>C</u>=CH_2), 164.6, 168.8 (C=O). LRMS m/z (ES^-): 142.1 $[M-H]^-$.



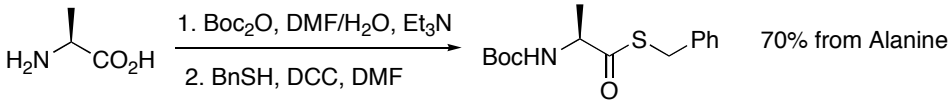
(**6**) Clear oil. IR (υ_{max}, film): 3384, 2929, 2490, 1647, 1423, 1072, 880, 813. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 2.72 (6H, s, 2 × NCH_3), 2.75 (6H, s, 2 × NCH_3), 3.92 (3H, s, CO_2CH_3), 7.86-7.89 (2H, m, ArH), 7.93-7.96 (1H, m, ArH). ^{13}C NMR (125 MHz, $CDCl_3$): δ = 36.99, 37.02 (NMe_2), 53.4 (CO_2<u>C</u>H_3), 124.3, 128.1, 133.9 (d, J_{CP} = 5.7), 136.1 (d, J_{CP} = 4.8), 137.8 (d, J_{CP} = 4.8), 146.8 (Ar), 165.4 (C=O). ^{31}P NMR (202 MHz, $CDCl_3$): 37.55. HRMS m/z (ESI^+): Found 370.0593 $[M+Na]^+$; $C_{12}H_{18}N_3O_5PSNa$ requires 370.0597.

(L)-Cysteine methyl ester hydrochloride



MeOH (75 mL) was added to a flame-dried 250 mL round bottom flask and cooled to 0 ºC. Acetyl chloride (29.3 mL, 413 mmol) was added slowly to the stirred solution and then stirred an additional 20 min at 0 ºC to generate methanolic HCl. Cysteine (10.00 g, 82.54 mmol) was added in one portion and the reaction stirred at room temperature for 14 hours under a balloon of nitrogen. The solvent was removed under reduced pressure to give cysteine methyl ester hydrochloride as an off-white solid (12.60 g, 89%) that was used without purification.

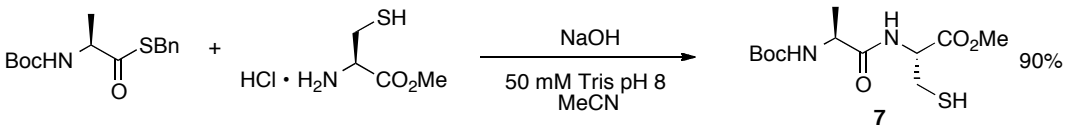
BocAlaSBn



L-Alanine (2.50 g, 28.0 mmol) was added to a 100 mL round bottom flask and suspended in DMF (10 mL). Et_3N (12.7 mL) and Boc_2O (7.96 g, 36.5 mmol) were then added sequentially and the reaction was stirred for 1 h (open air, RT). H_2O (10 mL) was then added to homogenize the reaction mixture and to the resulting solution was added an additional portion of Boc_2O (1.20 g, 5.50 mmol). The reaction was stirred for an additional 30 minutes at which time the reaction was diluted with $NaHCO_3$ (sat., 100 mL). The reaction mixture was washed with Et_2O (3 × 100 mL). The organic wash was discarded. The aqueous layer was cooled to 0 ºC and acidified to ~ pH 2 with 10 M HCl before extracting with EtOAc (3 × 100 mL). The combined organic layers were washed successively with H_2O (150 mL) and brine (150 mL) before drying over $MgSO_4$. After filtration, the product was concentrated under reduced pressure to give BocAlaOH which was carried on to the next step without further purification. R_f (1% AcOH in EtOAc) = 0.2. BocAlaOH (28 mmol, crude from above) was dissolved in DMF (25 mL) and placed under argon. Benzylthiol (7.23 mL, 61.6 mmol) was added to the stirred solution followed by DCC (7.51 g, 36.4 mmol) and an additional 20 mL of DMF. The reaction was stirred for 2 hours at room temperature before diluting with Et_2O (300 mL) and H_2O (250 mL). After separation, the organic layer was washed successively with 150 mL each of $NaHCO_3$ (sat.), H_2O, and brine. The organic layer was dried ($MgSO_4$), filtered, and the solvent removed under reduced pressure. The product was purified by column chromatography (100% petrol to 15% EtOAc in petrol) to give the titled compound as a white solid (R_f = 0.6). The solid was recrystalized from CH_2Cl_2/petrol to give BocAlaSBn as bright white needles (5.80 g, 70% from Ala). m.p. = 79-80 ºC. $[\alpha]^{20}_D$ = -25.1 (c =

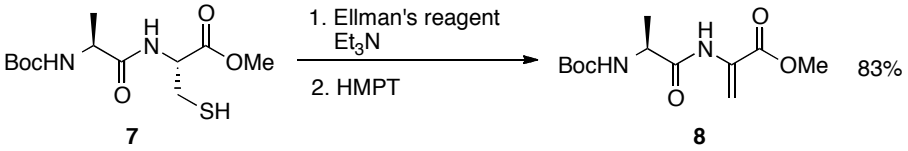
1.0, $CHCl_3$). IR (υ_{max}, KBr): 3375, 2980, 1689, 1505, 1341, 1383, 1250, 1164; ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 1.38 (3H, d, *J* = 7.3, C<u>H</u>$_3$), 1.46 (9H, s, Boc), 4.11 (2H, app. d, *J* = 4.8, SC<u>H</u>$_2$Ph), 4.43 (1H, m, Hα), 5.04 (1H, d, *J* = 7.6, NH), 7.32-7.22 (5H, m, Ar). ^{13}C NMR (100 MHz): δ = 18.7 (CH_3), 28.3 (Boc), 33.2 (S<u>C</u>H$_2$Ph), 56.2 (Cα), 80.3 (Boc), 127.3, 128.6, 128.9 (CH_{Ar}), 137.1 ($4°_{Ar}$), 154.9, 201.5 (2 × C=O). HRMS m/z (ESI+): Found 318.1134 $(M+Na)^+$; $C_{15}H_{21}NO_3SNa$ requires 318.1140. Analysis for $C_{15}H_{21}NO_3S$: C: 60.99, H: 7.17, N: 4.74; Found: C: 60.86; H: 7.32; N: 4.66.

BocAlaCysOMe (7)



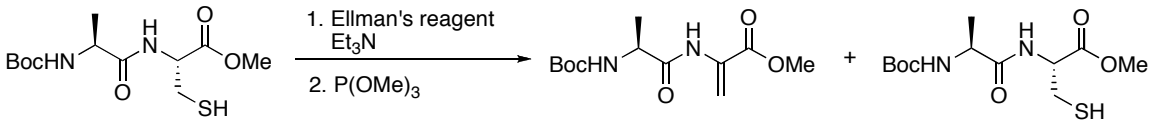
L-Cysteine methyl ester hydrochloride (5.00 g, 29.1 mmol) was added to a 100 mL, 2-neck round bottom flask and dissolved in 15 mL of pH 8.0 TRIS (50 mM). The solution was cooled to 0 ºC and 5.0 mL of 5.82 M NaOH was added dropwise. BocAlaSBn (2.50 g, 8.46 mmol) was added as a solution in MeCN (25 mL). The resulting solution (pH 9.0, pH paper) was stirred at room temperature for 5 hours after which time TLC indicated complete comsumption of thioester (R_f = 0.4, 10% EtOAc in petrol) and formation of the ligated product **7** (R_f = 0.5, 50% EtOAc in petrol). Tributylphosphine (1.05 mL, 4.26 mmol) was added to reduce any disulfide and after 15 min the reaction was diluted with Et_2O (250 mL) and H_2O (150 mL). The layers were separated and the aqueous layer was extracted with Et_2O (100 mL). The combined organics were washed with H_2O (2 × 150 mL) and brine (150 mL) and then dried ($MgSO_4$), filtered, and concentrated under reduced pressure. Purification by column chromatography (10% EtOAc in petrol to 50% EtOAc in petrol) provided BocAlaCysOMe (**7**) as white crystals (2.59 g, 90%). m.p. = 101-102 ºC. $[\alpha]^{20}_D$ = -1.9 (c = 1.0, $CHCl_3$). IR (υ_{max}, KBr): 3387, 3298, 2979, 2565, 1746, 1700, 1653, 1503, 1443, 1390, 1362, 1308. ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 1.35 (3H, d, *J* = 7.1, C<u>H</u>$_{3\ Ala}$), 1.41 (9H, s, Boc), 1.50 (1H, t, *J* = 8.7, SH), 3.03-2.90 (2H, m, C<u>H</u>$_2$SH), 3.75 (3H, s, CO_2C<u>H</u>$_3$), 4.21 (1H, br. m, Hα $_{Ala}$), 4.82 (1H, ddd, *J* = 7.8, 4.3, 4.0, Hα $_{Cys}$), 5.24 (1H, d, *J* = 6.1, NH $_{Ala}$), 7.13 (1H, d, *J* = 5.8, NH $_{Cys}$). ^{13}C NMR (100 MHz): δ =17.9 ($CH_{3\ Ala}$), 26.6 (<u>C</u>H$_2$SH), 28.3 (Boc), 50.1 (Cα $_{Ala}$), 52.8 (CO_2<u>C</u>H$_3$), 53.7 (Cα $_{Cys}$), 80.1 (Boc), 155.5, 170.3, 172.7 (3 × C=O). HRMS m/z (ESI^+): Found 329.1142 $(M+Na)^+$; $C_{12}H_{22}N_2O_5SNa$ requires 329.1147. Analysis for $C_{12}H_{22}N_2O_5S$: C: 47.04, H: 7.24, N: 9.14; Found: C: 47.05, H: 7.25, N: 9.09.

BocAlaDhaOMe (8) using HMPT



7 **8**

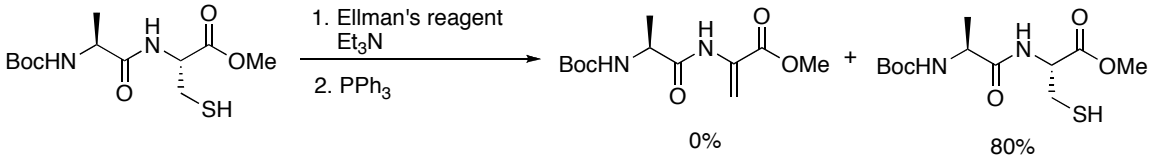
BocAlaCysOMe (**7**) (200 mg, 0.065 mmol) was added to a 25 mL pear shaped flask and dissolved in DMF (5 mL). NEt_3 (0.20 mL, 1.4 mmol) was added to the stirred solution followed immediately by Ellman's reagent (0.028 g, 0.071 mmol). The reaction mixture was stirred, open air, for 5 minutes at room temperature at which time TLC (50% EtOAc in petrol) revealed consumption of BocAlaCysOMe (R_f = 0.5) and formation of cysteine-*p*-NO_2-*m*-CO_2H-phenylthiol disulfide (base line). HMPT (0.015 mL, 0.083 mmol) was added by microsyringe and the reaction was stirred for an additional 15 minutes at room temperature. TLC (50% EtOAc in petrol) indicated formation of BocAlaDhaOMe (R_f = 0.7). The reaction was diluted with Et_2O (150 mL) and washed sequentially with 1 M HCl (75 mL), H_2O (75 mL), and brine (75 mL). The organic layer was dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The resulting residual mixture was purified by column chromatography (40% EtOAc in petrol) to afford BocAlaDhaOMe as a white solid (150 mg, 83%). m.p. = 64-65 ºC. $[\alpha]^{20}_D$ = -53.9 (c = 1.1, $CHCl_3$). IR (υ_{max}, film): 3332, 2980, 1690, 1523, 1442, 1367, 1249, 1167. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.40 (3H, d, *J* = 7.3, Me_{Ala}), 1.46 (9H, s, Boc), 3.84 (3H, s, $CO_2\underline{Me}$), 4.26 (1H, br. app. s, $H\alpha_{Ala}$), 5.01 (1H, br. app. s, NH_{Ala}), 5.91 (1H, d, *J* = 1.26, C=C$\underline{H}$H), 6.60 (1H, app s, C=CH$\underline{H}$), 8.46 (1H, br. s, NH_{Dha}). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 17.9 (Me_{Ala}), 28.2 (Boc), 50.9 ($C\alpha_{Ala}$), 52.9 ($CO_2\underline{Me}$), 80.4 (Boc), 109.2 (C=$\underline{C}H_2$), 130.8 ($\underline{C}$=CH_2), 155.5, 164.2, 171.5 (3 × C=O). HRMS m/z (ESI$^+$): Found 295.1262 $[M+Na]^+$; $C_{12}H_{20}N_2O_5Na$ requires 295.1264. Anal. Calcd. for $C_{12}H_{20}N_2O_5$: C, 52.93; H, 7.40; N, 10.29. Found C, 52.62; H, 7.28; N, 10.16.



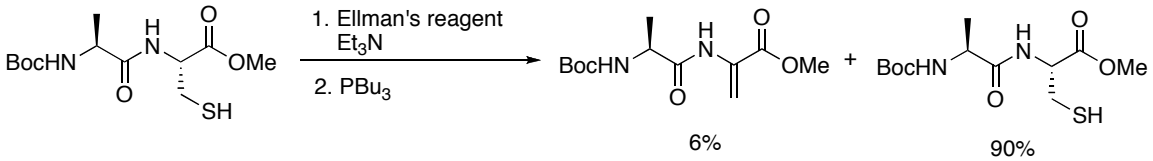
3% 33%

BocAlaCysOMe (100 mg, 0.326 mmol) was added to a flame dried 25 mL round bottom flask and dissolved in anhydrous DMF (3 mL). Ellman's reagent (142 mg, 0.358 mmol) was added along with NEt_3 (0.37 mL, 2.7 mmol). The reaction mixture was stirred for 15 min, open to air, at room temperature. After this time, TLC (EtOAc) revealed complete consumption of BocAlaCysOMe. $P(OMe)_3$ (0.080 mL, 0.68 mmol) was then added. The reaction was monitored by TLC. Only a small product spot was observed and mostly reduced cysteine was reformed.

After 15 minutes, the mixture was concentrated under reduced pressure and purified by flash column chromatography (30% EtOAc in petrol) to provide BocAlaDhaOMe (3 mg, 3%) and recovered BocAlaCysOMe (29 mg, 33%). Spectroscopic data was consistent with that reported above.



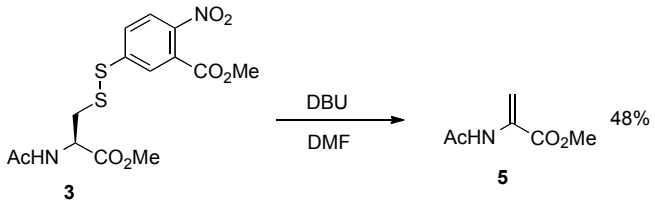
BocAlaCysOMe (20 mg, 0.065 mmol) was added to a 25 mL round bottom flask and dissolved in DMF (5 mL). NEt_3 (0.15 mL, 1.1 mmol) was added to the stirred solution. Ellman's reagent (28 mg, 0.071 mmol) was then added, and a dark red color was observed. The reaction flask was rinsed with DMF (1.5 mL) and the reaction stirred for 5 min after which time PPh_3 (23 mg, 0.088 mmol) was added. The mixture was stirred open air at room temperature for an additional 15 minutes. The solution was diluted in Et_2O (150 mL) and H_2O (100 mL) and transferred to a separatory funnel. The organic layer was separated, washed with 1 M HCl (200 mL), H_2O (100 mL) and brine (100 mL), dried over $MgSO_4$ and filtered. The solvent removed under reduced pressure. Purification of the resulting mixture by flash column chromatography provided recovered BocAlaCysOMe (16 mg, 80%). No BocAlaDhaOMe was observed. Spectroscopic data was consistent with that reported above.



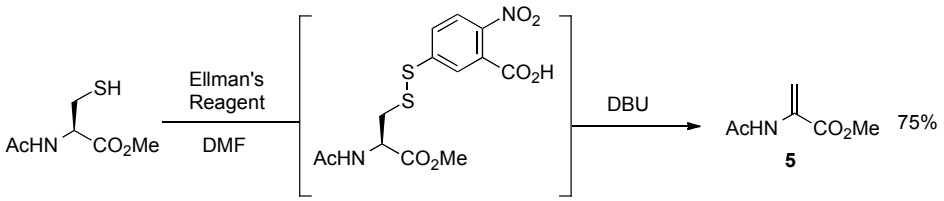
BocAlaCysOMe (20 mg, 0.065 mmol) was added to a 25 mL round bottom flask and dissolved in DMF (3 mL). NEt_3 (0.15 mL, 1.1 mmol) was added followed by addition of Ellman's reagent (28 mg, 0.071 mmol). The solution turned dark red. The reaction flask was rinsed with DMF (2 mL) and the reaction mixture stirred for 5 min. After this time PBu_3 (0.021 mL, 0.084 mmol) was added. The reaction was stirred for an additional 15 minutes after which the mixture was diluted with Et_2O (150 mL) and 1 M HCl (100 mL) and transferred to a separatory funnel. The organic layer was separated, washed with H_2O (100 mL), brine (100 mL), dried over $MgSO_4$ and filtered. The solvent was removed under reduced pressure and the resulting mixture was purified by column chromatography (40% EtOAc in petrol) to give the recovered BocAlaCysOMe (18 mg,

90%) and the BocAlaDhaOMe (1 mg, 6%). Spectroscopic data was consistent with that reported above.

Base-mediated conversion of cysteine to dehydroalanine

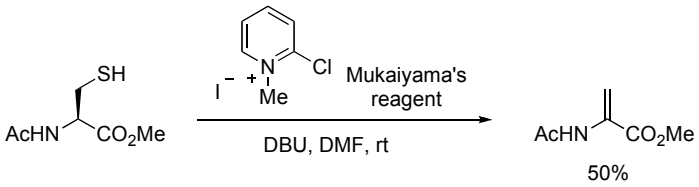


Disulfide **3** (40 mg, 0.10 mmol) was added to a 25 mL round bottom flask and dissolved in 3 mL of DMF. DBU (0.03 mL, 0.21 mmol) was added and the reaction turned deep red. The reaction was stirred 20 minutes at room temperature and then diluted with EtOAc (100 mL). The organic layer was washed sequentially with 1 M HCl (100 mL), H_2O (100 mL), and brine (100 mL). The organic layer was dried ($MgSO_4$), filtered, and concentrated under reduced pressure. The resulting residue was purified by column chromatography (65% EtOAc in petrol) to yield AcDhaOMe (**5**) (7 mg, 48%). Spectroscopic data was identical to that reported above.



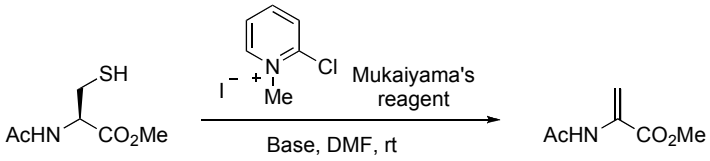
AcCysOMe (50 mg, 0.28 mmol) was added to a 25 mL round bottom flask and dissolved in 5 ml of DMF. Ellman's reagent (123 mg, 0.31 mmol) was added and the reaction stirred 10 minutes at room temperature. DBU (0.42 mL, 2.8 mmol) was then added, upon which the reaction mixture turned dark red. After 3 minutes, TLC revealed formation of AcDhaOMe. After a total of 10 minutes of reaction time the mixture was diluted with EtOAc (150 mL) and then washed sequentially with 1M HCl (2 × 100 mL) and brine (100 mL). The organic layer was then dried ($MgSO_4$), filtered, and concentrated under reduced pressure. The resulting residue was purified by column chromatography (65% EtOAc in petrol) to yield AcDhaOMe (**5**) (30 mg, 75%). Spectroscopic data was identical to that reported above.

Mukaiyama's Reagent in the Conversion of Cysteine to Dehydroalanine



DBU (2.50 mL, 17.0 mmol) was added dropwise to a dispersion of *N*-acetyl-L-cysteine methyl ester (1.00 g, 5.64 mmol) and 2-chloro-1-methylpyridinium iodide (1.63 g, 6.4 mmol) in dry and degassed DMF (5.6 mL) at room temperature under an argon atmosphere. After 5 minutes at the same temperature, the reaction mixture was diluted with EtOAc and washed with 1 M aqueous HCl and water. The combined organic layers were dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by column chromatography (from 1:1 EtOAc/petrol to EtOAc) to afford AcDhaOMe (404 mg, 50%) as a colorless crystalline solid. Spectroscopic data is consistent with that previously reported.[2] m.p. = 40-41 ºC. IR (υ_{max}, film): 1730, 1677, 1636, 1519, 1440, 1372, 1235, 1203, 1171. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 2.14 (3H, s, NHAc), 3.85 (3H, s, CO_2CH_3), 5.89 (1H, d, *J* = 1.6, C=C<u>H</u>H), 6.60 (1H, app. s, C=CH<u>H</u>), 7.73 (1H, br. s, NH). ^{13}C NMR (125 MHz, $CDCl_3$): δ = 24.7 (NHAc), 53.0 (CO_2CH_3), 108.7 (C=<u>C</u>H_2), 130.8 (<u>C</u>=CH_2), 164.6, 168.8 (C=O). LRMS m/z (ES^-): 142.1 $[M-H]^-$.

Additional experiments were carried out (see table below) to show that extended reaction time did not improve the yield (Entries 1 and 2). Also, conversion to the Mukaiyama adduct is necessary and DBU does not eliminate cysteine to dehydroalanine directly (Entries 3 and 4).

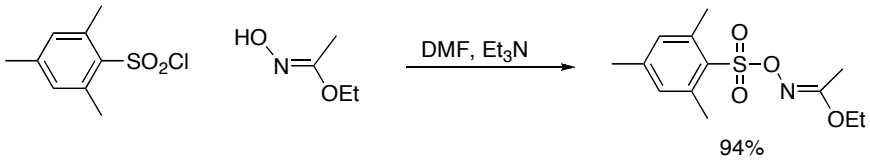


Entry[a]	Base (eq)	Reaction Conditions	Product (%)[b]
1	DBU (3)	Mukaiyama's reagent, DMF, rt, 2 h	40
2	DBU (3)	Mukaiyama's reagent, DMF, rt, 5 min	50
3[c]	DBU (3)	DMF, rt, 5 min	<5
4[d]	Et_3N (1.1), DBU (3)	Mukaiyama's reagent, DMF, rt, 30 min	40

(a) General conditions: AcCysOMe (1 eq), Mukaiyama's reagent (1.1 eq), and base (3 eq) in dry and degassed DMF (1 mL/mmol) unless otherwise indicated. (b) Yield refers to isolated product. (c) The reaction was conducted in the absence of Mukaiyama's reagent. (d) Et_3N was used in the pre-formation of the corresponding Mukaiyama's adduct prior to the addition of DBU.

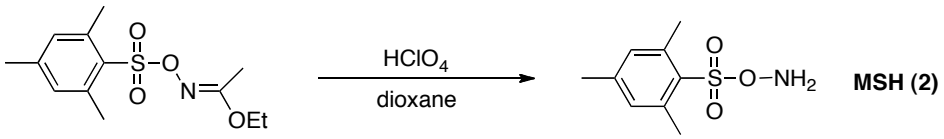
MSH Reactivity Study: Substrate Preparation

MSH Synthesis



94%

Ethyl *N*-hydroxyacetimidate (2.36 g, 22.9 mmol) was added to a 100 mL round bottom flask and dissolved in DMF (15 mL). Et_3N (6.4 mL, 46 mmol) was added and the solution was cooled to 0 ºC. 2-Mesitylenesulfonyl chloride (5.00 g, 22.9 mmol) was added as a solid in several portions. The reaction was stirred vigorously at 0 ºC for 30 minutes. A white solid was formed over the course of the reaction. After this time, the reaction was diluted with EtOAc (150 mL) and then washed with H_2O (3 × 100 mL) and brine (100 mL). The organic layer was dried ($MgSO_4$), filtered, and concentrated under reduced pressure to give a pale yellow oil that crystallized on storage at -20 ºC (6.11 g, 94%). No purification was required. m.p. = 51-53 ºC (Lit[3] = 57-58 ºC). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.19 (3H, t, *J* = 7.1, $OCH_2\underline{C}H_3$), 2.04 (3H, s, CH_3 imidate), 2.31 (3H, s, Me_{Ar}), 2.64 (6H, s, Me_{Ar}), 3.90 (2H, q, *J* = 7.1, $OC\underline{H}_2CH_3$), 6.97 (2H, s, CH_{Ar}). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 14.0 ($OCH_2\underline{C}H_3$), 14.9 (CH_3 imidate), 21.1 (Me_{Ar}), 22.8 (Me_{Ar}), 63.6 ($O\underline{C}H_2CH_3$), 130.3, 131.5, 140.7, 143.3 (4 × C_{Ar}), 169.7 (C=N).

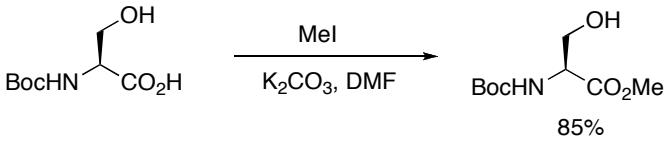


70%

The MSH precursor (6.11 g, 21.4 mmol) was dissolved in dioxane (8 mL) at room temperature and then cooled to 0 ºC. Perchloric acid (4 mL, 70% solution) was added dropwise over 2 minutes. The reaction was stirred vigorously at 0 ºC and thickens and solidifies over the course of 5 minutes. The reaction was incubated for an additional 5 minutes to ensure complete hydrolysis and then poured onto 50 g of ice. Et_2O (100 mL) and H_2O (100 mL) were both added to the quenched mixture and then transferred to a separatory funnel. The organic layer was separated and then dried / neutralized for 2 minutes with K_2CO_3. The solid was then removed by filtration and rinsed with Et_2O. The filtrate was concentrated carefully under reduced pressure to about 50 mL total volume. The solution was poured into a beaker with 150 mL ice cold petrol. MSH crystals formed immediately. The beaker was incubated at 0 ºC for 30 minutes to complete the

crystallization. The crystals were isolated by filtration and then dried under high vacuum for 15 minutes. The white needles (3.21 g, 70%) were stored in a plastic tube, sealed only with wax film. m.p. = 90-91 ºC (Lit[3] = 95-96 ºC). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.33 (3H, s, Me_{Ar}), 2.65 (6H, s, Me_{Ar}), 5.56 (2H, br. s, NH_2), 7.01 (2H, s, CH_{Ar}). ^{13}C NMR (50 MHz, $CDCl_3$): δ = 21.0 (Me_{Ar}), 22.6 (Me_{Ar}), 129.0, 131.6, 140.9, 143.7 (4 × C_{Ar}).

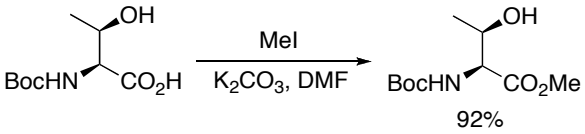
BocSerOMe



85%

(L)-BocSerOH (1.00 g, 4.87 mmol) was added to a 25 mL round bottom flask and dissolved in DMF (10 mL). K_2CO_3 (1.01 g, 7.31 mmol) was added, followed by MeI (1.52 mL, 24.4 mmol). The reaction was stirred at room temperature for 5 hours. After this time, the reaction was diluted with EtOAc (200 mL) and then washed with H_2O (2 × 100 mL) and brine (100 mL). The organic layer was dried ($MgSO_4$), filtered, and concentrated under reduced pressure. The resulting oil (BocSerOMe) did not require purification (910 mg, 85%). $[\alpha]^{20}_D$ = +11.3 (c = 1.6, $CHCl_3$) (Lit[4]: +9.7 c = 4.3, $CHCl_3$). IR (υ_{max}, film): 3396, 2979, 1746, 1714, 1515, 1368, 1351, 1287, 1249, 1214. ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 1.40 (9H, s, Boc), 3.30 (1H, s, OH), 3.72 (3H, s, CO_2Me), 3.78-3.94 (2H, m, C<u>H</u>$_2$OH), 4.31 (1H, m, Hα), 5.61 (1H, d, *J* = 7.9, NH). ^{13}C NMR ($CDCl_3$, 50 MHz): δ = 28.2 (Boc), 52.4 (CO_2<u>Me</u>), 55.6 (Cα), 63.0 (<u>C</u>H$_2$OH), 80.1 (Boc), 155.7, 171.4 (2 × C=O). LRMS m/z (ESI$^+$): 242.1 $[M+Na]^+$.

BocThrOMe

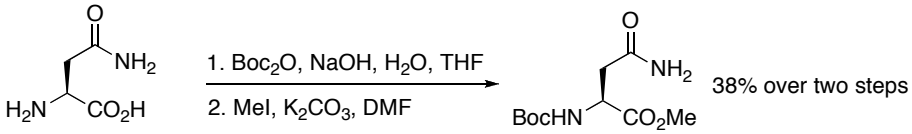


92%

(L)-BocThrOH (1.00 g, 4.56 mmol) was added to a 10 mL round bottom flask and suspended in DMF (10 mL). K_2CO_3 (946 mg, 6.84 mmol) was added to the stirred mixture, followed by iodomethane (0.85 mL, 13.7 mmol). The reaction was stirred at room temperature for 30 minutes. After this time, the reaction was diluted with EtOAc (100 mL) and then washed sequentially with sat. $NaHCO_3$ (50 mL) and brine (2 × 50 mL). The organic layer was dried ($MgSO_4$), filtered, and concentrated under reduced pressure. The resulting oil was sufficiently pure to not require purification (981 mg, 92%). Spectroscopic data was consistent with that previously reported.[5] $[\alpha]^{20}_D$ = -10.1 (c = 1.0, CH_2Cl_2) (Lit[5]: -8.77, c = 0.75, CH_2Cl_2). IR (υ_{max}, film): 3436, 2979, 2935,

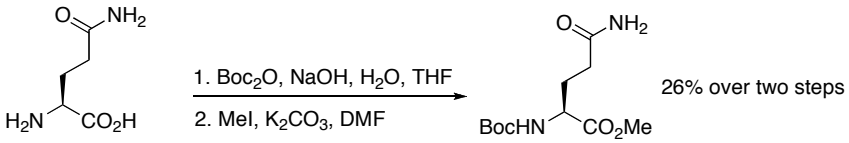
1748, 1718, 1509, 1367, 1166. ^{1}H (400 MHz, $CDCl_3$): δ = 1.21 (3H, d, *J* = 6.3,CH_3), 1.41 (9H, s, Boc), 2.87 (1H, d, *J* = 4.3, OH), 3.73 (3H, s, CO_2Me), 4.21 (1H, m, Hα), 4.26 (1H, m, Hβ), 5.44 (1H, d, *J* = 8.6, NH). ^{13}C (100 MHz, $CDCl_3$): δ = 19.8 (CH_3), 28.2 (Boc), 52.3 (CO_2Me), 60.3 (Cα), 67.9 (Cβ), 79.9 (Boc), 156.1, 172.0 (2 × C=O). LRMS m/z (ESI^+): 256.1 $[M+Na]^+$.

BocAsnOMe



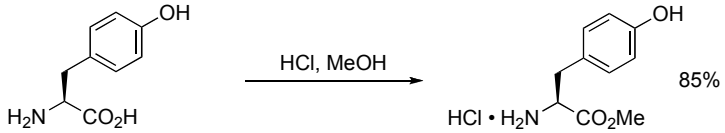
(L)-Asparagine (2.00 g, 15.1 mmol) was added to a 100 mL round bottom flask and suspended in H_2O (20 mL). NaOH (1.27 g, 31.8 mmol) was added to the flask and the mixture was stirred until all material was dissolved. Boc_2O (3.97 g, 18.2 mmol) was then added as a solution in THF (20 mL). The reaction was stirred at room temperature for 1 hour and then quenched by the addition of 1M HCl (50 mL). The product was extracted with EtOAc (3 × 40 mL). The combined organic layers were then washed with brine (70 mL), dried ($MgSO_4$), filtered, and concentrated under reduced pressure. The crude product (2.98 g) was carried on immediately to the next step. Accordingly, the crude (L)-BocAsnOH was dissolved in DMF (10 mL). To the stirred solution was added K_2CO_3 (2.66 g, 19.3 mmol) and methyl iodide (1.60 mL, 25.7 mmol). The reaction was stirred at room temperature for 1 hour and then diluted with EtOAc (100 mL). The mixture was washed sequentially with $NaHCO_3$ (50 mL sat. aq.), H_2O (50 mL), and brine (50 mL). The organic layer was then dried ($MgSO_4$), filtered, and concentrated under reduced pressure. The resulting residue was purified by column chromatography (EtOAc) to provide (L)-BocAsnOMe[6] as a white foam (1.42 g, 38% over two steps). $[\alpha]^{20}_D$ = -16.2 (c =0.5, MeOH). IR (υ_{max}, film): 3354, 2979, 1741, 1680, 1619, 1504, 1438, 1410, 1393, 1367, 1348, 1317, 1286, 1251, 1222, 1167, 1120. ^{1}H (400 MHz, $CDCl_3$): δ = 1.42 (9H, s, Boc), 2.71 (1H, ABX system, *J* = 15.9, 4.3, Hβ), 2.91 (1H, ABX system, *J* = 15.9, 4.8, Hβ′), 3.73 (3H, s, CO_2Me), 4.50 (1H, m, Hα), 5.79 (1H, d, *J* = 8.3, NHBoc), 6.06-6.09 (2H, app. d, *J* = 9.9, NH_2). ^{13}C (100 MHz, $CDCl_3$): δ = 28.2 (Boc), 37.3 (Cβ), 50.2 (Cα), 52.6 (CO_2Me), 80.0 (Boc), 155.6, 172.1, 172.5 (3 × C=O). LRMS m/z (ESI^+): 269.1 $[M+Na]^+$.

BocGlnOMe



(L)-Glutamine (1.00 g, 6.84 mmol) was added to a 100 mL round bottom flask and suspended in H_2O (10 mL). NaOH (574 mg, 14.4 mmol) was added and the mixture was stirred until all material was dissolved. Boc_2O (1.79 g, 8.20 mmol) was added as a solution in THF (10 mL). The reaction was stirred at room temperature for 1 hour and then quenched by the addition of 1M HCl (50 mL). The product was extracted with EtOAc (3 × 40 mL). The combined organic layers were then washed with brine (100 mL), dried ($MgSO_4$), filtered, and concentrated under reduced pressure. The crude product (1.68 g) was carried on immediately to the next step. Accordingly, the crude BocGlnOH was dissolved in DMF (14 mL). K_2CO_3 (1.24 g, 8.97 mmol) was added followed by methyl iodide (0.74 mL, 12 mmol). The reaction was stirred for 30 minutes at room temperature and then diluted with EtOAc (100 mL). The organic fraction was washed sequentially with $NaHCO_3$ (50 mL sat. aq.) and brine (2 × 50 mL). After drying ($MgSO_4$), the organic layer was filtered and concentrated under reduced pressure. The resulting residue was purified by column chromatography (EtOAc) to afford (L)-BocGlnOMe as a waxy solid (460 mg, 26% over two steps). Spectroscopic data was consistent with that previously reported.[7] $[\alpha]^{20}_D$ = -21.2 (c = 0.5, MeOH). IR (υ_{max}, film): 3346, 2978, 2248, 1700, 1682, 1620, 1520, 1454, 1392, 1367, 1218, 1166, 1097, 1054, 1028. ^{1}H (400 MHz, $CDCl_3$): δ = 1.37 (9H, s, Boc), 1.87-1.92 (1H, m, Hβ), 2.05-2.15 (1H, m, Hβ′), 2.24-2.34 (2H, m, Hγ), 3.68 (3H, s, CO_2Me), 4.21-4.26 (1H, m, Hα), 5.56 (1H, d, *J* = 7.8, NHBoc), 6.05 (1H, s, CONHH), 6.40 (1H, s, CONHH). ^{13}C (100 MHz, $CDCl_3$): δ = 28.1 (Boc), 28.3 (Cβ), 31.6 (Cγ), 52.4 (CO_2Me), 52.9 (Cα), 79.9 (Boc), 155.7, 172.7, 174.7 (3 × C=O). LRMS m/z (ESI^+): 283.1 $[M+Na]^+$.

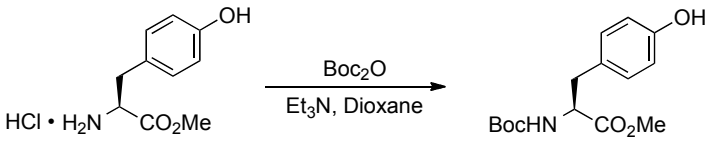
(L)-Tyrosine methyl ester hydrochloride



A 250 mL round bottom flask was flame dried and placed under an atmosphere of nitrogen. Anhydrous MeOH (80 mL) was added to the flask and cooled to 0 ºC. Acetyl chloride (193 mmol, 13.74 mL) was added slowly with stirring to generate methanolic HCl. After all of the acetyl chloride was added, the solution was stirred an additional 15 minutes at 0 ºC. (L)-Tyrosine

(7.00 g, 38.6 mmol) was added as a solid in one portion. The ice bath was removed and the reaction stirred at room temperature for 12 hours. After this time, volatiles were removed under reduced pressure to give the crude product as an off-white solid. The solid was triturated by stirring rapidly in 50 mL of boiling acetone for 10 minutes. After cooling to room temperature, the flask was cooled on ice for several minutes. The resulting micro-crystals were isolated by filtration and washed with 50 mL of cold acetone. The crystals were then dried under vacuum (7.65 g, 85% yield). m.p. = 172-174 ºC, decomposes at 190 ºC (Lit[8]: 190-192, decomp.). $[\alpha]^{20}_{D}$ = +69.8 (c = 3.0, pyridine) (Lit[9]: +72.0, c = 3.0, pyridine). IR (υ_{max}, KBr): 3343, 2879, 1958, 1744, 1614, 1592, 1515, 1448, 1397, 1349, 1226, 1141, 1107, 1060. ^{1}H NMR (400 MHz, MeOD): δ = 3.10-3.22 (2H, ABX system, *J* = 14.3, 7.2, 6.1, C<u>H</u>$_2$Ar), 3.82 (3H, s, $CO_2C\underline{H}_3$), 4.28 (1H, app t. *J* = 6.7, Hα), 6.81 (2H, d, *J* = 8.5, ArH), 7.10 (2H, d, *J* = 8.5, ArH). ^{13}C NMR (50 MHz, MeOD): δ = 36.7 ($\underline{C}H_2Ar$), 53.7, 55.6 (Cα and $CO_2\underline{C}H_3$), 117.0, 125.8, 131.7, 158.4 (Ar), 170.6 (C=O). LRMS m/z (ESI$^-$): 194.1 $[M-H]^-$.

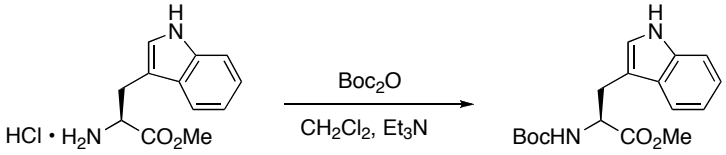
BocTyrOMe



84%

L-Tyrosine methyl ester hydrochloride (3.23 g, 16.6 mmol) was added to a 250 mL round bottom flask and suspended in dioxane (50 mL). To the stirred suspension was added Et_3N (4.65 mL, 33.1 mmol). Boc_2O (4.70 g, 21.5 mmol) was added in one portion and the reaction was stirred at room temperature. After 5 hours of reaction time, the reaction was diluted with EtOAc (150 mL) and washed with H_2O (150 mL). The aqueous layer was extracted with EtOAc (2 × 150 mL) and the combined organic layers were washed with brine (200 mL), dried ($MgSO_4$), filtered, and concentrated under reduced pressure. The resulting residue was purified by column chromatography by eluting first with 15% EtOAc in petrol and then 50% EtOAc in petrol. The product was isolated as a white solid (4.13 g, 84% yield). m.p. = 96-99 ºC (Lit.[10] = 102 ºC). $[\alpha]^{20}_{D}$ = +41.2 (c = 1.0, $CHCl_3$) (Lit[11]: +53.1, c = 1.0, $CHCl_3$), IR (υ_{max}, KBr): 3370, 2979, 1687, 1516, 1445, 1368, 1224, 1166. ^{1}H (200 MHz, $CDCl_3$): δ = 1.42 (9H, s, Boc), 3.00 (2H, ABX system, *J* = 5.8, 6.0, 13.6, C<u>H</u>$_2$Ar), 3.71 (3H, s, $CO_2C\underline{H}_3$), 4.54 (1H, m, Hα), 5.06 (1H, d, *J* = 4, NH), 6.48 (1H, br s, OH), 6.73 (2H, d, *J* = 4.0, CH_{Ar}), 6.96 (2H, d, *J* = 4.0, CH_{Ar}). ^{13}C (50 MHz, $CDCl_3$): δ = 28.3 (Boc), 37.6 ($\underline{C}H_2Ar$), 52.4 ($CO_2\underline{C}H_3$), 54.6 (Cα), 80.3 (Boc), 115.5, 127.3, 130.3, 155.3, 155.4, 172.7 (C_{Ar} and C=O). LRMS m/z (ESI$^-$): 294.1 $[M-H]^-$.

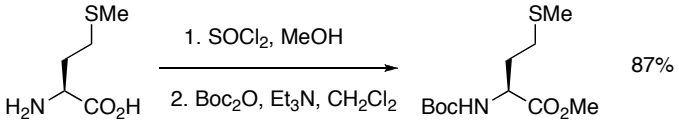
BocTrpOMe



94%

L-Tryptophan methyl ester hydrochloride (1.00 g, 3.93 mmol) was added to a 100 mL round bottom flask and suspended in CH_2Cl_2 (20 mL). Et_3N (1.64 mL, 11.8 mmol) was added and the mixture was stirred until all material was dissolved. Boc_2O (943 mg, 4.32 mmol) was added and the reaction was stirred at room temperature for 4 hours. After this time, the reaction was diluted with CH_2Cl_2 (100 mL) and washed with 1M HCl (100 mL). The organic layer was dried ($MgSO_4$), filtered, and concentrated under reduced pressure. The resulting solid was recrystallized (CH_2Cl_2/petrol) and isolated as small white needles (1.179 g, 94%). m.p. = 143-144 ºC (Lit[12]: 151-152 ºC). $[\alpha]^{20}_D$ = +55.7 (c = 1.0, $CHCl_3$) (Lit[4]: +47.5, c = 0.05, $CHCl_3$). IR (υ_{max}, film): 3357, 2978, 1739, 1697, 1503, 1457, 1437, 1367, 1249, 1218, 1167. 1097, 1063. ^{1}H NMR ($CDCl_3$, 200MHz): δ = 1.45 (9H, s, Boc), 3.30 (2H, d, *J* = 5.4, C<u>H</u>$_2$Ar), 3.68 (3H, s, CO_2<u>Me</u>), 4.62-4.72 (1H, m, Hα) 5.13 (1H, d, *J* = 7.9, NH), 6.97 (1H, s, CH_{Ar}), 7.09-7.24 (2H, m, CH_{Ar}), 7.32-7.37 (1H, m, CH_{Ar}), 7.55-7.59 (1H, m, CH_{Ar}), 8.40 (1H, s, NH). ^{13}C NMR ($CDCl_3$, 50 MHz): δ = 27.9 (<u>C</u>H$_2$Ar), 28.3 (Boc), 52.2 (CO_2<u>Me</u>), 54.2 (Hα), 79.9 (Boc), 109.9 (4^o_{Ar}), 111.2, 118.6, 119.5, 122.0, 122.8 (5 × CH_{Ar}), 127.6, 136.1 (2 × 4^o_{Ar}), 155.3, 172.8 (2 × C=O). LRMS m/z (ESI$^+$): 341.2 $[M+Na]^+$.

BocMetOMe

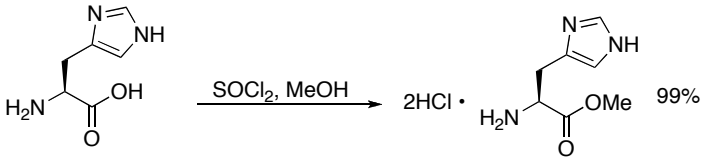


87%

To a flame dried flask under nitrogen was added MeOH (30 mL). The stirred solution was cooled to 0 ºC before $SOCl_2$ (3.70 mL, 50.67 mmol) was added dropwise. The solution was stirred 10 min at 0 ºC before methionine (5.04 g, 33.8 mmol) was added in one portion. The reaction was stirred at room temperature overnight after which time the volatiles were removed under reduced pressure to give the crude methionine methyl ester hydrochloride as a yellow-white solid. This solid was dissolved in CH_2Cl_2 (150 mL) and cooled to 0 ºC. Et_3N (17.5 mL, 126 mmol) was added carefully followed by Boc_2O (13.6 g, 62.5 mmol). After stirring for 7 hours at room temperature, the reaction was diluted with CH_2Cl_2 (100mL) and washed with H_2O (2 × 100 mL).

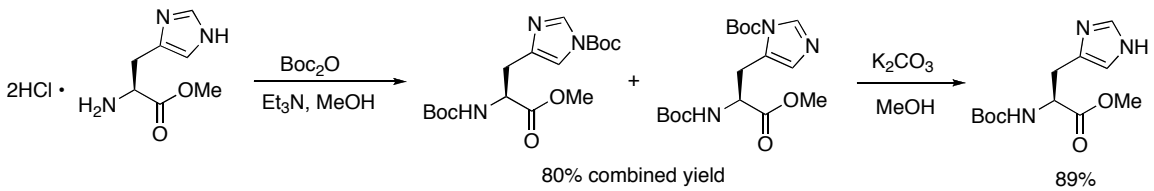
The organic layer was dried ($MgSO_4$), filtered, and the solvent removed under reduced pressure. The product (R_f = 0.6 in 20% EtOAc in petrol) was purified by column chromatography (20% EtOAc in petrol) to afford the titled compound as a clear oil (7.75 g, 87% yield). $[\alpha]^{20}_D$ = -29.5, c = 1.0, MeOH (Lit[13] = -34.0, c = 1.0, MeOH). IR (υ_{max}, film): 3359, 2977, 2920, 1744, 1716, 1515, 1438, 1391, 1366, 1251, 1221, 1167. ^{1}H NMR (400 MHz $CDCl_3$): δ = 1.39 (9H, s, Boc), 1.88 (1H, m, CH<u>H</u>CH_2SMe), 2.12-2.04 (4H, includes 3H, s, S<u>Me</u> and C<u>H</u>HCH_2SMe), 2.49 (2H, t, *J* = 8.0, C<u>H</u>$_2$SMe), 3.70 (3H, s, CO_2<u>Me</u>), 4.37 (1H, q, *J* = 7.1, Hα), 5.20 (1H, d, *J* = 7.1, NH). ^{13}C (100 MHz): δ =15.3 (S<u>C</u>H_3), 28.1 (Boc), 29.8 (<u>C</u>H_2CH_2SMe), 31.9 (<u>C</u>H_2SMe), 52.2 (CO_2<u>C</u>H_3), 52.6 (Cα), 79.8 (Boc), 155.2, 172.7 (2 × C=O). LRMS m/z (ESI$^+$): 286.1 $[M+Na]^+$.

(L)-Histidine methyl ester di-hydrochloride



L-Histidine (20.00 g, 128.9 mmol) was added to a 500 mL round bottom flask and dissolved in MeOH (100 mL, anhydrous). $SOCl_2$ (11.2 mL, 154.7 mmol) was added carefully by syringe under a balloon of nitrogen. After the addition of thionyl chloride, the flask was equipped with a condenser and heated at reflux for 10 hours. After this time the reaction was cooled to room temperature and the solvent removed under reduced pressure. The resulting solid was triturated by stirring vigorously in Et_2O (200 mL) for 20 minutes. The product was isolated by filtration as hygroscopic crystals (31.05 g, 99%). Spectroscopic data was consistent with that previously reported.[14] m.p. = 200-203 ºC. $[\alpha]^{20}_D$ = +10.3 (c = 2.0, H_2O). IR (υ_{max}, KBr): 2969, 2027, 1996, 1760, 1625, 1336, 1291, 1065. ^{1}H (400 MHz, D_2O): δ = 3.30-3.43 (2H, ABX system, *J* = 15.9, 7.1, 6.8, Hβ), 3.73 (3H, s, CO_2Me), 4.41 (1H, t, *J* = 6.8, Hα). 7.35 (1H, s, CH_{Ar}), 8.61 (1H, s, CH_{Ar}). ^{13}C (100 MHz, D_2O): δ = 25.3 (Cβ), 52.2 (Cα), 54.4 (CO_2Me), 118.7 (CH_{Ar}), 126.5 ($4^o{}_{Ar}$), 134.7 (CH_{Ar}), 169.2 (C=O). LRMS m/z (ESI$^+$): 170.1 $[M+H]^+$.

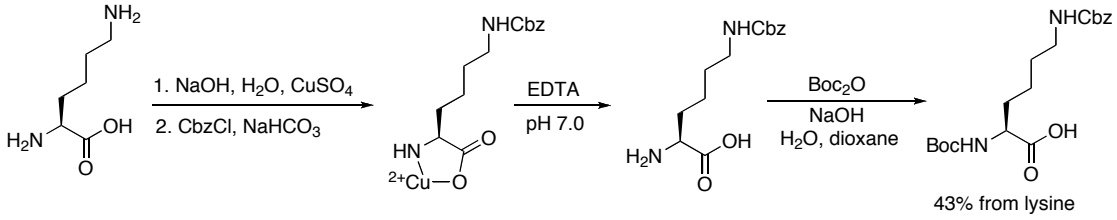
BocHisOMe



The following procedure was adapted from the literature.[14] L-Histidine methyl ester di-hydrochloride (1.00 g, 4.13 mmol) was added to a 100 mL round bottom flask and suspended in

MeOH (25 mL). Et_3N (2.9 mL, 20.7 mmol) was added slowly to the stirred suspension at room temperature. Boc_2O (2.07 g, 9.48 mmol) was added as a solid in one portion to the reaction flask. The reaction was stirred at room temperature for 2 hours and then diluted with EtOAc (200 mL). The mixture was washed sequentially with H_2O (150 mL) and brine (150 mL). The organic layer was dried ($MgSO_4$), filtered, and concentrated under reduced pressure. The resulting residue was purified by column chromatography (60% EtOAc in petrol) to give a mixture of regioisomers (1.24 g, 80% combined yield). Note: these isomers are separable by chromatography (R_f = 0.25, R_f = 0.20, 60% EtOAc in petrol), but were still combined since they converged to BocHisOMe on deprotection. Accordingly, a portion of the two regioisomers (906 mg, 2.45 mmol) was dissolved in MeOH (25 mL). K_2CO_3 (34 mg, 0.25 mmol) was added and the reaction mixture heated at reflux for 3 hours. TLC (EtOAc) revealed complete consumption of both regioiosomers. The reaction was filtered and concentrated under reduced pressure. The resulting residue was purified by column chromatography (10% MeOH in CH_2Cl_2) to provide BocHisOMe as a thick clear oil that crystallized upon standing (586 mg, 89%). m.p. = 135-136 ºC (Lit[15] = 128-129 ºC). $[\alpha]^{20}_D$ = -4.2 (c = 2.0, MeOH). IR (υ_{max}, film): 3300, 2978, 1743, 1710, 1512, 1437, 1392, 1367, 1288, 1252, 1215, 1167, 1055, 1024. ^{1}H (400 MHz, MeOD): δ = 1.41 (9H, s, Boc), 2.92-3.09 (2H, ABX system, *J* = 14.9, 8.6, 5.3, Hβ), 3.70 (3H, s, CO_2Me), 4.40 (1H, dd, *J* = 8.6, 5.3, Hα), 6.87 (1H, s, CH_{Ar}), 7.60 (1H, s, CH_{Ar}). ^{13}C (100 MHz, MeOD): δ = 28.8 (Boc), 30.4 (Cβ), 52.8 (CO_2Me), 55.4 (Cα), 80.8 (Boc), 118.3 (CH_{Ar}), 134.7 (4^o_{Ar}), 136.4 (CH_{Ar}), 157.9, 174.3 (2 × C=O). LRMS m/z (ESI^+): 270.1 $[M+H]^+$.

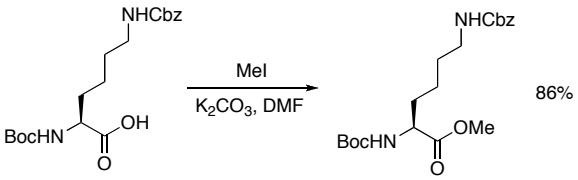
BocLys(Cbz)OH



This preparation was adapted from the literature.[16,17] L-Lysine (40.00 g, 219 mmol) was added to a 1 L flask and dissolved in H_2O (160 mL). The stirred solution was cooled to 0 ºC and NaOH (17.52 g, 438.0 mmol) was added as a solid. The reaction was stirred until all material was dissolved. $CuSO_4$ (17.48 g, 109.5 mmol) was prepared as a solution in H_2O (80 mL) in a separate flask and then poured into the solution of lysine. The resulting blue solution was cooled to 0 ºC and then $NaHCO_3$ (22.08 g, 262.8 mmol) was added. When all material was dissolved, benzylchloroformate (40.5 mL, 284.7 mmol) was poured into the reaction mixture at 0 ºC. The

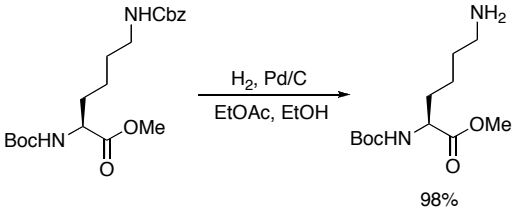
reaction was stoppered and stirred vigorously at 0 ºC for 1 hour and then at room temperature for 5 hours. After this time, the blue precipitate was isolated by filtration and washed with H_2O (200 mL). The solid was partially dried on the filter before washing with acetone (200 mL) to remove any excess CbzCl. The Lys(Cbz) copper complex was dried to a blue powder on the filter. In a 2 L beaker, a suspension of EDTA (70.4 g, 241 mmol) in H_2O was prepared and heated to 80 ºC. The Lys(Cbz) copper complex was added as a solid to the stirred solution of EDTA. A color change from lavender to bright aqua blue was observed as the EDTA copper complex formed. After all of the lysine derivative was added, the pH was adjusted to 7 by adding ~ 100 mL of 5M NaOH (the pH was checked using pH paper). At pH 7, a white precipitate was formed (Lys(Cbz)OH). The hot suspension was filtered and the solid isolated. The solid was then washed with H_2O (2 L) and then MeOH (500 mL). The white solid was dried on the filter and then under high vacuum. The crude material was used without purification in the next step. Accordingly, Lys(Cbz)OH (15.00 g, 53.51 mmol crude from above) was suspended in H_2O (50 mL). NaOH (4.49 g, 112 mmol) was added to the stirred suspension and the mixture was stirred at room temperature to dissolve. Once all material had dissolved, Boc_2O (11.68 g, 53.51 mmol) was added as a solution in 1,4-dioxane (50 mL). The reaction mixture was stirred vigorously at room temperature. Solid was formed during the reaction. After 4 hours, the reaction mixture was diluted with EtOAc (100 mL) and 1M HCl (100 mL). This mixture was stirred vigorously at room temperature to dissolve all material. The mixture was further diluted with EtOAc (100 mL) and 1M HCl (100 mL). The organic layer was separated, dried ($MgSO_4$), filtered, and concentrated under reduced pressure. The resulting residue was purified by column chromatography (10% MeOH in CH_2Cl_2), to give BocLys(Cbz)OH as a thick, clear oil (8.73 g, 43% from lysine). $[\alpha]^{20}_D$ = -5.6 (c = 3.3, MeOH). IR (υ_{max}, film): 3332, 2977, 2936, 1707, 1529, 1455, 1409, 1393, 1367, 1253, 1165, 1048, 1025. ^{1}H (400 MHz, DMSO): δ = 1.26-1.37 (13H, m, contains Boc, Hγ, Hδ), 1.53-1.65 (2H, m, Hβ), 2.98 (2H, q, *J* = 5.5, C$\underline{H}_2$NHCbz), 3.80-3.85 (1H, m, Hα), 5.00 (2H, s, C$\underline{H}_2$Ph), 6.90 (1H, d, *J* = 5.6, N$\underline{H}$Boc), 7.23 (1H, t, *J* = 5.5, N$\underline{H}$Cbz), 7.28-7.38 (5H, m, CH_{Ar}). ^{13}C (100 MHz, DMSO): δ = 22.9, 28.3, 29.1 (Cγ, Boc, Cδ), 30.7 (Cβ), 40.1 (C$\underline{H}_2$NHCbz), 53.7 (Cα), 65.2 (C$\underline{H}_2$Ph), 77.9 (Boc), 127.8 (2 × C), 128.4 (3 × CH_{Ar}) 137.3 (4^o_{Ar}), 155.6, 156.1, 174.6 (3 × C=O). LRMS m/z (ESI^+): 403.2 $[M+Na]^+$.

BocLys(Cbz)OMe



BocLys(Cbz)OH (7.23 g, 19.0 mmol) was added to a 100 mL round bottom flask and dissolved in DMF (40 mL). K_2CO_3 (5.25 g, 38.0 mmol) was added and the stirred solution was cooled to 0 ºC. iodomethane (1.05 mL, 24.7 mmol) was added and the reaction was stirred at 0 ºC for 5 minutes and then room temperature for 2 hours. After this time, the reaction was diluted with EtOAc (250 mL) and washed with H_2O (300 mL) and brine (2 × 300 mL). The organic layer was dried ($MgSO_4$), filtered, and concentrated under reduced pressure. The resulting residue was purified by column chromatography (45% EtOAc in petrol) to give BocLys(Cbz)OMe as a thick, clear oil (6.41 g, 86%). Spectroscopic data was consistent with that previously reported.[17] $[\alpha]^{20}_D$ = -22.5 (c = 1.0, MeOH). IR (υ_{max}, film): 3343, 2951, 2866, 1714, 1526, 1455, 1366, 1251, 1167, 1047, 1024. ^{1}H (400 MHz, $CDCl_3$): δ = 1.23-1.48 (13H, m, includes Boc, Hγ, Hδ), 1.53-1.78 (2H, m, Hβ), 3.07-3.11 (2H, q, *J* = 6.3, C<u>H</u>$_2$NHCbz), 3.63 (3H, s, CO_2Me), 4.16-4.23 (1H, m, Hα). 5.02 (2H, s, C<u>H</u>$_2$Ph), 5.26-5.35 (2H, m, N<u>H</u>Boc and N<u>H</u>Cbz), 7.20-7.30 (5H, m, CH_{Ar}). ^{13}C (100 MHz, $CDCl_3$): δ = 22.1, 28.0, 29.0 (Cγ, Boc, Cδ), 31.7 (Cβ), 40.2 (<u>C</u>H$_2$NHCbz), 51.9 (CO_2Me), 53.0 (Cα), 66.1 (<u>C</u>H$_2$Ph), 79.4 (Boc), 127.68, 127.74, 128.1 (3 × CH_{Ar}), 136.4 ($4°_{Ar}$), 155.3, 156.3, 173.3 (3 × C=O). LRMS m/z (ESI$^+$): 417.2 $[M+Na]^+$.

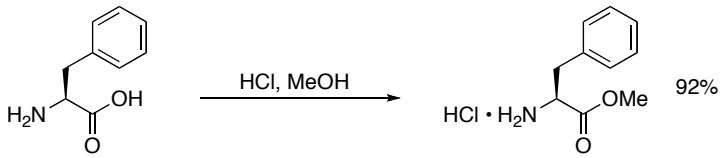
BocLysOMe



BocLys(Cbz)OMe (6.13 g, 15.5 mmol) was dissolved in a mixture of EtOAc (80 mL) and EtOH (20 mL). Palladium (5% wt. on carbon, 826 mg, 0.39 mmol) was added to the stirred solution. The flask was sealed and hydrogen was bubbled through the solution for 5 minutes. After 1 hour of stirring under an H_2 atmosphere (balloon), TLC (50% EtOAc in petrol) revealed complete consumption of starting material. The palladium and carbon was removed by filtration through a pad of celite. The solids were washed with an additional 100 mL of MeOH. The filtrate was concentrated under reduced pressure and the resulting residue was purified by column

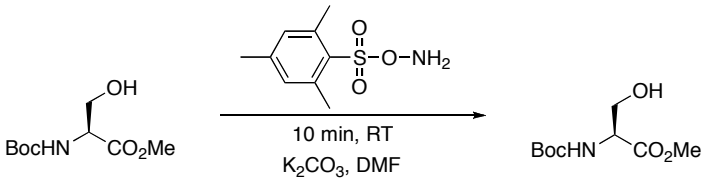
chromatography (10% MeOH in CH_2Cl_2 with 2% Et_3N). BocLysOMe was isolated as a thick, clear oil[17] (3.95 g, 98%). $[\alpha]^{20}_D$ = -29.0 (c = 1.0, MeOH). IR (υ_{max}, film): 3365, 2933, 1744, 1713, 1521, 1366, 1167, 1050, 1021. ^{1}H (400 MHz, $CDCl_3$): δ = 1.27-1.38 (15H, m, contains Boc, Hγ, Hδ, and NH_2), 1.46-1.76 (2H, m, Hβ), 2.59 (2H, t, *J* = 6.7, C<u>H</u>$_2$NH$_2$), 3.63 (3H, s, CO_2Me), 4.14-4.22 (1H, m, Hα), 5.29 (1H, d, *J* = 7.8, N<u>H</u>Boc). ^{13}C (100 MHz, $CDCl_3$): δ = 22.4, 28.1, 32.2, 32.8 (Cγ, Boc, Cδ, Cβ), 41.5 (<u>C</u>H$_2$NH$_2$), 51.9 (CO_2Me), 53.2 (Cα), 79.5 (Boc), 155.3, 173.2 (2 × C=O). LRMS m/z (ESI$^+$): 261.2 $[M+H]^+$.

(L)-Phenylalanine methyl ester hydrochloride



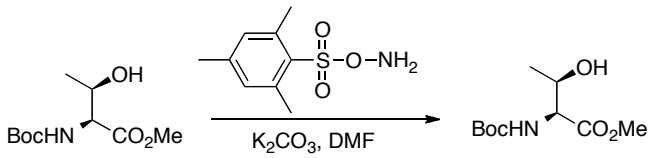
MeOH (20 mL) was added to a flame dried 100 mL round bottom flask under an atmosphere of argon. The flask was cooled to 0 ºC and acetyl chloride (4.30 mL, 60.5 mmol) was added carefully. The reaction was stirred at 0 ºC for 10 minutes to generate methanolic HCl. L-Phenylalanine (2.00 g, 12.1 mmol) was then added as a solid in one portion. The ice bath was removed and the reaction was stirred at room temperature for 12 hours. After this time, the solvent was removed under reduced pressure to give HCl•PheOMe as a white solid that did not require purification (2.41 g, 92%). m.p. = 150-151 ºC (Lit[18] = 158-159 ºC). $[\alpha]^{20}_D$ = +32.5 (c = 2.0, EtOH) (Lit[18]: +36.2, c = 2, EtOH). IR (υ_{max}, KBr): 2843, 2011, 1748, 1584, 1496, 1447, 1292, 1242, 1084, 1060, 1032. ^{1}H (400 MHz, D_2O): δ = 3.15 (2H, ABX system, *J* = 14.4, 7.6, 5.8, C<u>H</u>$_2$Ph), 3.70 (3H, s, CO_2<u>Me</u>), 4.29 (1H, dd, *J* = 7.3, 6.1, Hα), 7.14-7.31 (5H, m, ArH). ^{13}C (100 MHz, D_2O): δ = 35.9 (<u>C</u>H$_2$Ph), 53.9, 54.4 (CO_2Me, Cα), 128.5, 129.6, 129.7 (<u>C</u>H$_{Ar}$), 134.0 (4º$_{Ar}$), 170.4 (C=O). LRMS m/z (ESI$^+$): 180.1 $[M+H]^+$.

MSH Reactivity with Functionalized Amino Acids:



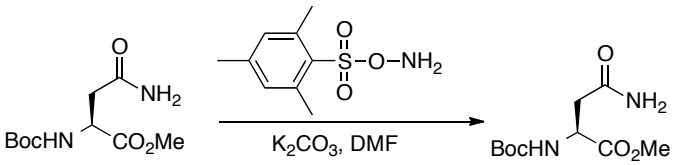
(No Reaction, 94% recovery)

BocSerOMe (50 mg, 0.23 mmol) was added to a 25 mL round bottom flask and dissolved in DMF (5 mL). K_2CO_3 (158 mg, 1.14 mmol) was added to the stirred solution, followed by MSH (124 mg, 0.58 mmol). The reaction was stirred at room temperature for 10 minutes and then diluted with EtOAc (100 mL). The solution was washed with H_2O (100 mL), dried ($MgSO_4$), filtered, and concentrated under reduced pressure. The resulting residue was purified by column chromatography (EtOAc) to provide unreacted BocSerOMe (47 mg, 94% recovery). Spectroscopic and analytical data was identical to that reported above for BocSerOMe.



(No Reaction, 96% recovery)

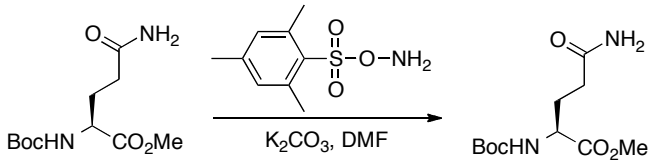
(L)-BocThrOMe (50 mg, 0.21 mmol) was added to a 10 mL round bottom flask and dissolved in DMF (4 mL). K_2CO_3 (148 mg, 1.07 mmol) was added, followed by MSH (113 mg, 0.53 mmol). The reaction was stirred at room temperature for 10 minutes. After this time, the mixture was diluted with EtOAc (60 mL) and washed sequentially with H_2O (60 mL) and brine (60 mL). The organic layer was dried ($MgSO_4$), filtered, and concentrated under reduced pressure. The resulting residue was purified by column chromatography to afford 48 mg of unreacted BocThrOMe (96% recovery). Spectroscopic data was identical to that reported above for BocThrOMe.



(No Reaction, 100% recovery)

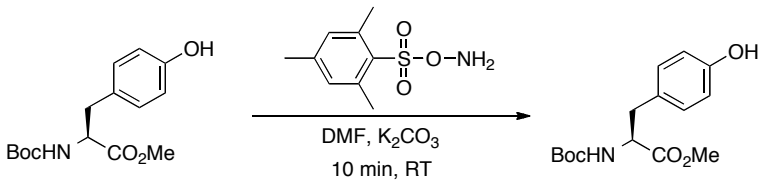
(L)-BocAsnOMe (20 mg, 0.081 mmol) was added to a 5 mL round bottom flask and dissolved in DMF (0.5 mL). K_2CO_3 (56 mg, 0.41 mmol) was added, followed by MSH (44 mg, 0.20 mmol). The reaction was stirred 10 minutes at room temperature and then purified directly by column

chromatography (EtOAc). Unreacted (L)-BocAsnOMe was recovered in quantitative yield (20 mg, 100% recovery). Spectroscopic data was identical to that reported above.



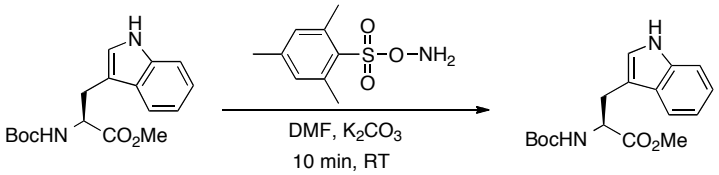
(No Reaction, 95% Recovery)

(L)-BocGlnOMe (20 mg, 0.077 mmol), was added to a 5 mL round bottom flask and dissolved in DMF (1 mL). K_2CO_3 (53 mg, 0.38 mmol) was added followed by MSH (42 mg, 0.19 mmol). The reaction was stirred for 10 minutes at room temperature and then purified directly by column chromatography (10% MeOH in CH_2Cl_2). Unreacted (L)-BocGlnOMe was recovered (19 mg, 95% recovery). Spectroscopic data was identical to that reported above.



(No Reaction, 90% recovery)

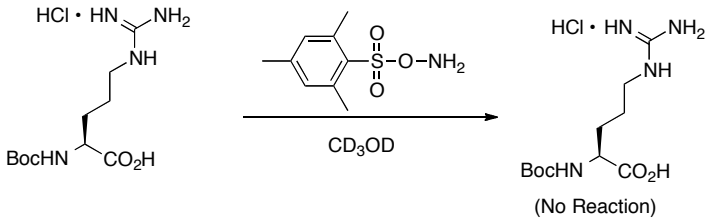
BocTyrOMe (100 mg, 0.34 mmol) was added to a 25 mL round bottom flask and dissolved in DMF (5 mL). K_2CO_3 (234 mg, 1.69 mmol) was added, followed by MSH (183 mg, 0.85 mmol). The reaction was stirred at room temperature for 10 minutes and then diluted with EtOAc (100 mL). The solution was washed with H_2O (100 mL), brine (100 mL), and then dried over $MgSO_4$. After filtration, the solvent was concentrated under reduced pressure. The resulting residue was purified by column chromatography (50% EtOAc in petrol) to afford 90 mg of unreacted BocTyrOMe (90% recovery). Spectroscopic and analytical data were identical to that reported above for the starting material.



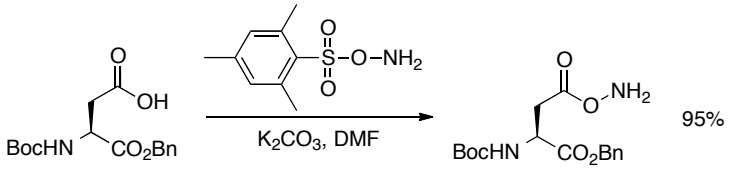
(No Reaction, 94% Recovery)

BocTrpOMe (50 mg, 0.16 mmol) was added to a 25 mL round bottom flask and dissolved in DMF (5 mL). K_2CO_3 (109 mg, 0.79 mmol) was added to the stirred solution, followed by MSH (84 mg, 0.39 mmol). The reaction was stirred at room temperature for 10 minutes and then diluted with EtOAc (100 mL). The solution was washed with H_2O (100 mL) and brine (100 mL).

The organic layer was dried ($MgSO_4$), filtered, and concentrated under reduced pressure. The resulting residue was purified by column chromatography (60% EtOAc in petrol), providing unreacted BocTrpOMe (47 mg, 94% recovery). Spectroscopic and analytical data was identical to that reported above for BocTrpOMe.

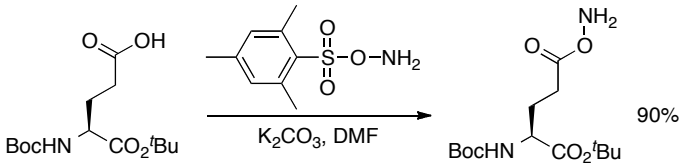


BocArgOH hydrochloride (5.0 mg, 0.016 mmol) was added to a glass vial and dissolved in 300 μL CD_3OD. MSH (9.0 mg, 0.042 mmol) was dissolved in 300 μL CD_3OD and then added to the solution of BocArgOH. The reaction was incubated at room temperature for 10 minutes and then analyzed directly by ^1H NMR and ESI-MS. No reaction was observed and only unreacted BocArgOH was detected in both NMR and ESI-MS.



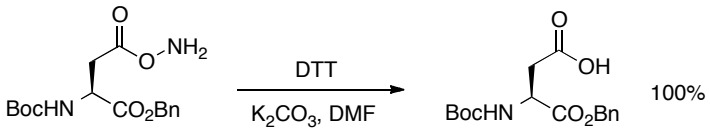
9

(L)-BocAspOBn (Fluka, 20 mg, 0.062 mmol) was added to a 5 mL round bottom flask and dissolved in DMF (1 mL). K_2CO_3 (51 mg, 0.37 mmol) and MSH (33 mg, 0.16 mmol) were added sequentially to the stirred solution. The reaction was stirred for 10 minutes at room temperature and then purified directly by column chromatography (10% MeOH in CH_2Cl_2). A single product was isolated (R_f = 0.7, 10% MeOH in CH_2Cl_2) and determined to be the product of carboxyamination (clear oil, 20 mg, 95%). $[\alpha]^{20}_D$ = -20.9 (c = 1.0, MeOH). IR (υ_{max}, film): 3314, 2978, 2934, 2361, 1744, 1713, 1500, 1367, 1342, 1289, 1247, 1215, 1163. ^1H (500 MHz, $CDCl_3$): δ = 1.43 (9H, s, Boc), 2.95 (2H, ABX system, *J* = 16.7, 4.7, Hβ), 4.65 (1H, m, Hα), 5.20 (2H, AB quart., *J* = 12.3, CH_2Ph), 5.49 (1H, d, *J* = 7.9, NHBoc), 6.28 (2H, br. s, NH_2), 7.34-7.39 (5H, m, ArH). ^{13}C (125 MHz, $CDCl_3$): δ = 28.2 (Boc), 35.2 (Cβ), 50.0 (Cα), 67.6 (CH_2Ph), 80.4 (Boc), 128.4, 128.5, 128.6 (CH_{Ar}), 135.1 ($4°_{Ar}$), 155.2, 170.5, 171.5 (3 × C=O). HRMS m/z (ESI^+): Found 361.1369 $[M+Na]^+$; $C_{16}H_{22}N_2O_6Na$ requires 361.1370.



10

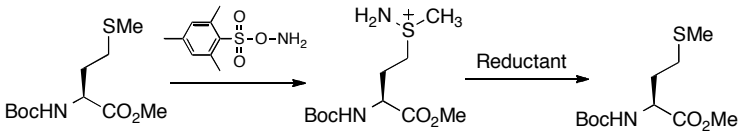
(L)-BocGluO^{t}Bu (Fluka, 20 mg, 0.066 mmol) was added to a 5 mL round bottom flask and dissolved in DMF (0.5 mL). K_2CO_3 (55 mg, 0.40 mmol) was added to the stirred solution, followed by MSH (36 mg, 0.17 mmol). The reaction was stirred 10 minutes at room temperature and then purified directly by column chromatography (EtOAc). A single product was isolated (R_f = 0.6, EtOAc) and identified as the product of carboxyamination (clear oil, 19 mg, 90%). $[\alpha]^{20}_D$ = -22.5 (c = 1.0, MeOH). IR (υ_{max}, film): 3313, 2978, 2934, 2360, 2342, 1734, 1714, 1558, 1508, 1455, 1392, 1367, 1250, 1154. ^{1}H (500 MHz, $CDCl_3$): δ = 1.44 (9H, s), 1.47 (9H, s) (2 × ^{t}Bu), 1.91-1.97 (1H, m Hβ), 2.15-2.24 (1H, m, Hβ′), 2.37-2.49 (2H, m, Hγ), 4.20-4.25 (1H, m, Hα), 5.10 (1H, d, *J* = 6.9, NHBoc), 6.36 (2H, br. s, NH_2). ^{13}C (125 MHz, $CDCl_3$): δ = 28.0 (^{t}Bu), 28.1 (Cβ), 28.3 (^{t}Bu), 28.5 (Cγ), 53.1 (Cα), 79.9, 82.4 (2 × ^{t}Bu), 155.4, 171.1, 173.6 (3 × C=O). HRMS m/z (ESI$^+$): Found 341.1680 $[M+Na]^+$; $C_{14}H_{26}N_2O_6Na$ requires 341.1683.

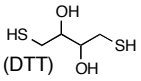


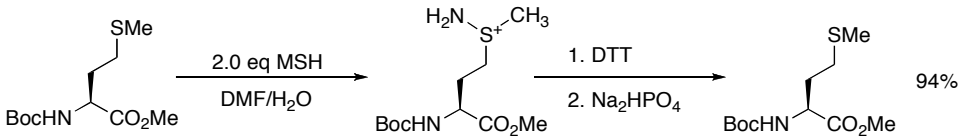
9

Carboxyaminated BocAspOBn (**9**) (20 mg, 0.059 mmol) was added to a 5 mL round bottom flask and dissolved in DMF (1 mL). K_2CO_3 (33 mg, 0.24 mmol) and dithiothreitol (DTT, 18 mg, 0.12 mmol) were added to the stirred solution. After 5 minutes, TLC and ESI-MS indicated that all aminated product had been reduced to BocAspOBn. After 30 minutes of total reaction time, the mixture was purified directly by column chromatography (EtOAc). BocAspOBn (deaminated) was isolated in quantitative yield (19 mg, 100%). Spectroscopic data was identical to the commercial material (Fluka).

Summary of Reaction of MSH with Methionine and Attempted Reduction Back to Thioether:

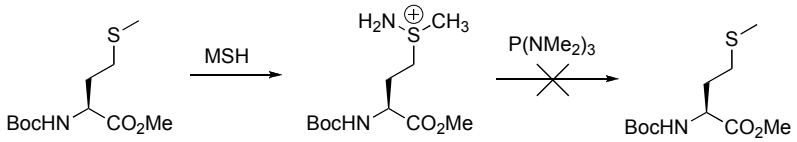


Entry	Reductant	Conditions	Yield BocMetOMe
1	 (DTT)	Na_2HPO_4, DMF, H_2O, 2h, RT	94%
2	none (control)	Na_2HPO_4, DMF, H_2O, 2h, RT	20%
3	$P(NMe_2)_3$	DMF, H_2O, 3h, RT	0%
4	PBu_3	DMF, H_2O, 3h, RT	0%
5	DTT (5 mol%), PBu_3	Na_2HPO_4, DMF, H_2O, 3h, RT	17%
6	Zn powder	DMF, H_2O, AcOH, 8h, RT	0%
7	$Na_2S_2O_6$	Na_2CO_3, DMF, H_2O, 2h, RT	11%
8	sodium ascorbate	DMF, H_2O, 3h, RT	0%

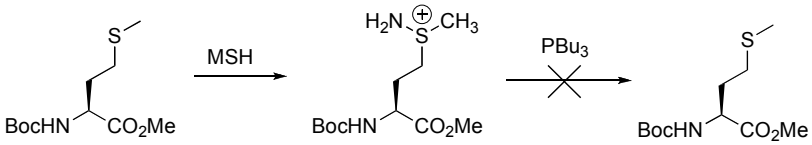


BocMetOMe (245 mg, 0.93 mmol) was added to a 50 mL round bottom flask and then dissolved in DMF (5 mL). The solution was stirred vigorously while H_2O (5 mL) was added by pipette. MSH (400 mg, 1.86 mmol) was added to the solution in one portion and the cloudy suspension homogenized after 30 seconds of stirring. After 5 minutes, TLC analysis (30% EtOAc in petrol) revealed complete consumption of BocMetOMe. All material was located on the baseline. After 20 minutes of stirring, DTT (1.43 g, 1.86 mmol) was added as a solid. TLC analysis revealed no change after 1 hour of stirring. After 1 hour of total reaction time, $NaHPO_4 \cdot 12H_2O$ (3.33 g, 9.30 mmol) was added to give a saturated solution of phosphate salts. After 2 hours of total reaction time (1 hour with base), TLC (30% EtOAc in petrol) revealed the regeneration of BocMetOMe. A final hour of reaction time revealed no further change. The reaction was then diluted with Et_2O (150 mL) and H_2O (150 mL) and separated. The organic layer was washed sequentially with H_2O (150 mL) and brine (150 mL), dried ($MgSO_4$), filtered, and concentrated under reduced pressure.

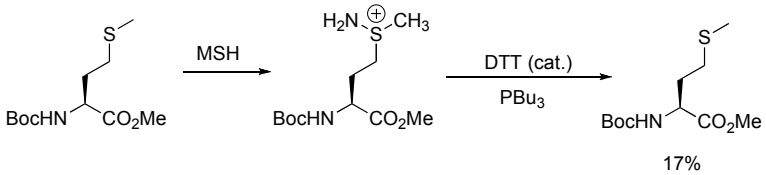
The product was purified by column chromatography (30% EtOAc in petrol) to give recovered BocMetOMe (230 mg, 94%). A similar experiment without DTT returned only 20% of BocMetOMe.



BocMetOMe (100 mg, 0.38 mmol) was added to a 25 mL round bottom flask and dissolved in a mixture of DMF (5 mL) and H_2O (2 mL). MSH (123 mg, 0.57 mmol) was added at room temperature and the reaction stirred for 5 minutes. TLC revealed complete consumption of starting material. HMPT (0.14 mL, 0.76 mmol) was added and the reaction monitored periodically over the course of three hours. No regeneration of starting material was observed.

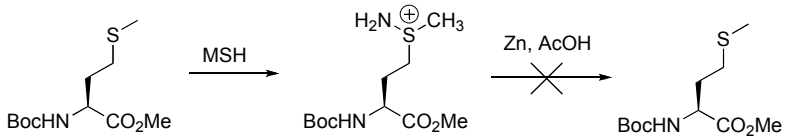


BocMetOMe (100 mg, 0.38 mmol) was added to a 25 mL round bottom flask and dissolved in a mixture of DMF (5 mL) and H_2O (2 mL). MSH (123 mg, 0.57 mmol) was added at room temperature and the reaction stirred for 5 minutes. TLC revealed complete consumption of starting material. PBu_3 (0.23 mL, 0.95 mmol) was added and the reaction stirred 10 min at room temperature. An additional 0.5 mL of PBu_3 was added and the reaction stirred for 3 hours. No BocMetOMe was regenerated.

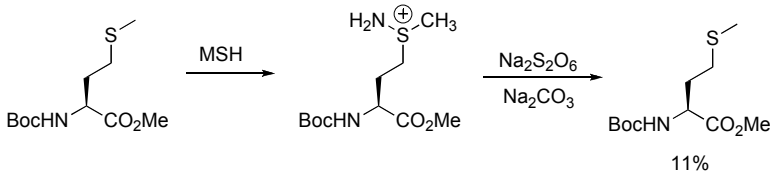


BocMetOMe (100 mg, 0.38 mmol) was added to a 25 mL round bottom flask and dissolved in a mixture of DMF (5 mL) and H_2O (2 mL). MSH (123 mg, 0.57 mmol) was added at room temperature and the reaction stirred for 5 minutes. TLC revealed complete consumption of starting material. Na_2CO_3 (201 mg, 1.9 mmol), PBu_3 (0.19 mL, 0.76 mmol), and DTT (3 mg, 0.02 mmol) were added sequentially. After 10 minutes, an additional 3 mg of DTT (0.02 mmol) was added. After 3 hours at room temperature, the reaction was diluted with Et_2O (150 mL) and then washed with H_2O (100 mL) and brine (100 mL). The organic layer was then dried, filtered, and

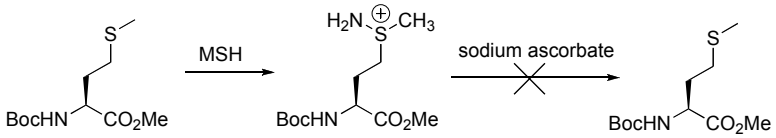
concentrated under reduced pressure. The resulting residue was purified by column chromatography (35% EtOAc in petrol) to give 17 mg (17%) of recovered BocMetOMe. Spectral data was identical to the starting material.



BocMetOMe (100 mg, 0.38 mmol) was added to a 25 mL round bottom flask and dissolved in a mixture of DMF (5 mL) and H_2O (2 mL). MSH (123 mg, 0.57 mmol) was added at room temperature and the reaction stirred for 5 minutes. TLC revealed complete consumption of starting material. AcOH (2.0 mL) and zinc powder (100 mg) were then added sequentially and the reaction stirred vigorously at room temperature. No BocMetOMe was detected after an hour so an additional 500 mg of zinc powder was added. No BocMetOMe was regenerated after 8 hours of reaction.



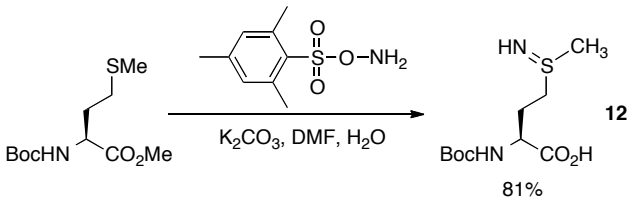
BocMetOMe (100 mg, 0.38 mmol) was added to a 25 mL round bottom flask and dissolved in a mixture of DMF (5 mL) and H_2O (2 mL). MSH (123 mg, 0.57 mmol) was added at room temperature and the reaction stirred for 5 minutes. TLC revealed complete consumption of starting material. Na_2CO_3 (121 mg, 1.14 mmol) was added followed by sodium dithionate (330 mg, 1.9 mmol). After 2 hours at room temperature, some starting material was regenerated. The reaction was diluted with Et_2O (150 mL) and H_2O (100 mL). The organic layer was separated and then washed with H_2O (2 × 100 mL) and brine (100 mL). The organic layer was dried, filtered, and then concentrated under reduced pressure. The resulting residue was purified by column chromatography (35% EtOAc in petrol) to give 11 mg of BocMetOMe (11%). Spectral data was identical to the starting material.



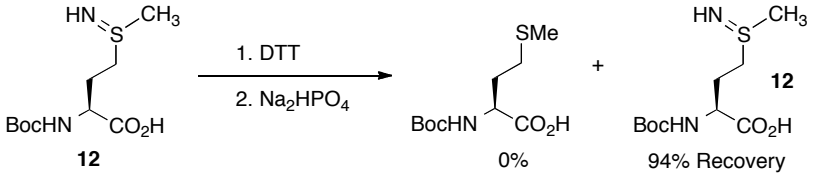
BocMetOMe (100 mg, 0.38 mmol) was added to a 25 mL round bottom flask and dissolved in a mixture of DMF (5 mL) and H_2O (2 mL). MSH (123 mg, 0.57 mmol) was added at room

temperature and the reaction stirred for 5 minutes. TLC revealed complete consumption of starting material. Sodium ascorbate (376 mg, 1.9 mmol) was added and the reaction followed by periodically by TLC. After three hours no BocMetOMe was regenerated.

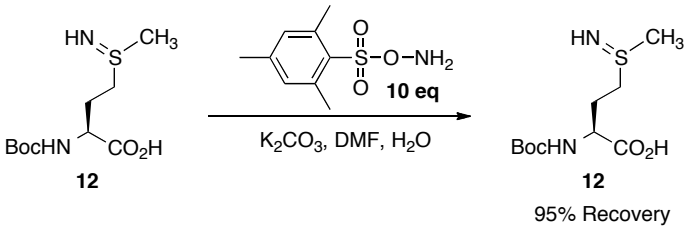
Isolation of Methionine Sulfilimine:



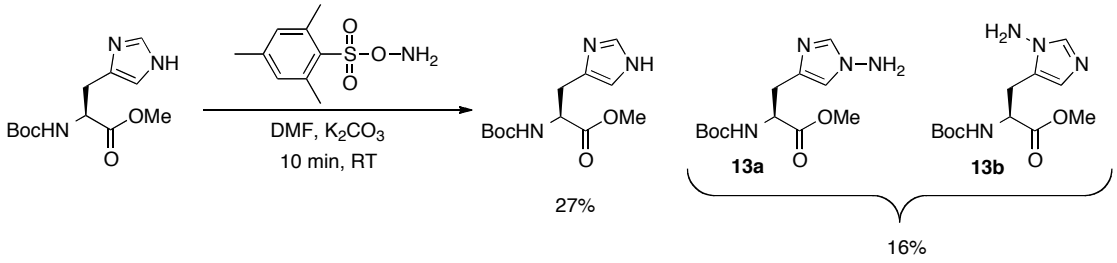
MSH (84 mg, 0.39 mmol) was added to a stirred solution of BocMetOMe (52 mg, 0.20 mmol) in 1:1 DMF/H_2O (4.8 mL) at room temperature. After 5 minutes, K_2CO_3 (135 mg, 0.980 mmol) was added and the mixture stirred at the same temperature for 2.5 h. The reaction was concentrated under reduced pressure and purified directly by column chromatography (7:2:1 EtOAc/MeOH/H_2O) to afford sulfilimine **12** (42 mg, 81%) as mixture of diastereomers as a white solid. Note that the ester was hydrolyzed to the free acid under these conditions. IR (υ_{max}, KBr): 3226, 3212, 3072, 2978, 2929, 2857, 1765, 1723, 1168, 1017. ^{1}H NMR (500 MHz, CD_3OD): δ = 1.45 (9H, s, Boc), 1.93-2.25 (2H, m, Hβ), 2.65 (3H, s, CH_3), 2.78-2.93 (2H, m, Hγ), 4.19 (1H, m, Hα). ^{13}C NMR (125 MHz, CD_3OD, both diastereomers reported where peaks resolved): δ = 26.7, 26.8 (Cβ), 28.8 (Boc), 38.26, 38.29 (CH_3), 51.28, 51.33 (Cγ), 54.6, 54.9 (Cα), 81.0 (Boc), 157.88, 157.92 (Boc C=O), 176.5 (C=O). HRMS m/z (ESI$^+$): Found 287.1036 $[M+Na]^+$; $C_{10}H_{20}N_2O_4$ SNa requires 287.1036.



Dithiothreitol (DTT) (16.2 mg, 0.105 mmol) was added to a stirred solution of BocMetOH sulfilimine **12** (13.9 mg, 0.053 mmol) and Na_2HPO_4 (75.8 mg, 0.526 mmol) in 1:1 DMF/H_2O (1.3 mL) at room temperature for 15 h. The crude was concentrated under reduced pressure and purified by column chromatography (7:2:1 EtOAc/MeOH/H_2O) to provide unreacted **12** (13.1 mg, 94%) as mixture of diastereomers as a white solid. Spectroscopic data was identical to that reported above.

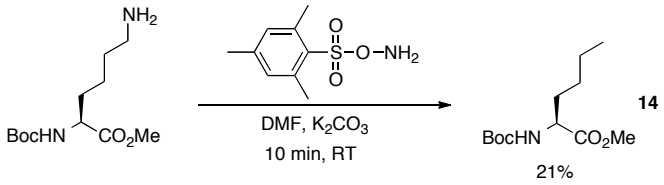


MSH (68.4 mg, 0.318 mmol, 10 equiv.) was added to a stirred solution of BocMetOH sulfilimine **12** (8.4 mg, 0.032 mmol) in 1:1 DMF/H_2O (768 μL) at room temperature. After 5 minutes, K_2CO_3 (22.0 mg, 0.159 mmol) was added and the mixture stirred at the same temperature for 3 days. The crude was concentrated under reduced pressure and purified by column chromatography (7:2:1 EtOAc/MeOH/H_2O) to recover **12** (8.0 mg, 95%) as a white solid. Spectroscopic and MS data was identical to that reported above for **12**. Note that the product of any *C*-terminal carboxyamination was hydrolyzed over the course of the reaction and no amination was observed at sulfur.

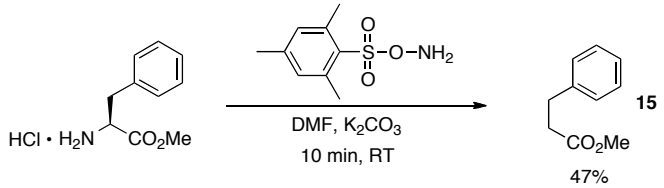


BocHisOMe (500 mg, 1.86 mmol) was added to a 25 mL round bottom flask and dissolved in DMF (8 mL). K_2CO_3 (1.29 g, 9.30 mmol) was added, followed by MSH (450 mg, 2.09 mmol). The reaction was stirred at room temperature for 10 minutes and then diluted with EtOAc (200 mL). The solution was washed with H_2O (200 mL) and brine (200 mL). The organic layer was dried ($MgSO_4$), filtered, and concentrated under reduced pressure. TLC (10% MeOH in EtOAc) revealed 3 products. Careful separation by column chromatography (10% MeOH in EtOAc) provided unreacted BocHisOMe (134 mg, 27%) and a mixture of regioisomers of the amination products **13a** and **13b** (87 mg). One of the regioisomers was isolated by a second purification by column chromatography (10% MeOH in EtOAc). Data for the isolated regioisomer: $[\alpha]^{20}_D$ = -1.0 (c = 0.5, MeOH). IR (υ_{max}, film): 3345, 3221, 2978, 1741, 1699, 1519, 1438, 1392, 1367, 1289, 1253, 1219, 1166. ^{1}H (400 MHz, $CDCl_3$): δ = 1.39 (9H, s, Boc), 3.02 (1H, dd, *J* = 15.0, 6.7, CHHAr), 3.19 (1H, dd, *J* = 15.0, 5.3, CHHAr), 3.75 (3H, s, CO_2Me), 4.62 (1H, m, Hα), 4.85 (2H,

s, NH_2), 5.26 (1H, app. s, N<u>H</u>Boc), 6.72 (1H, s, CH_{Ar}), 7.49 (1H, s, CH_{Ar}). ^{13}C (125 MHz, $CDCl_3$): δ = 26.5 (<u>C</u>H_2Ar), 28.2 (Boc), 52.6 (CO_2<u>Me</u>), 57.7 (Cα), 80.4 (Boc), 126.6 (CH_{Ar}), 127.0 ($4°_{Ar}$), 138.1 (CH_{Ar}), 135.2, 172.0 (2 × C=O). HRMS m/z (ESI^+): Found 285.1556 $[M+H]^+$; $C_{12}H_{21}N_4O_4$ requires 285.1557.



BocLysOMe (229 mg, 0.88 mmol) was added to a 50 mL round bottom flask and dissolved in DMF (10 mL). K_2CO_3 (608 mg, 4.40 mmol) was added, followed by MSH (473 mg, 2.2 mmol). The reaction was stirred 10 minutes at room temperature and then diluted with EtOAc (150 mL) and $NaHCO_3$ (sat. aq., 100 mL). The organic layer was separated, dried ($MgSO_4$), filtered, and concentrated under reduced pressure. TLC of the product mixture revealed that all BocLysOMe was consumed in the reaction and that a complicated product mixture had formed. The major product by TLC was isolated after careful purification by column chromatography (30% EtOAc in petrol). This material was assigned as Boc-norleucine methyl ester (**14**). (45 mg, 21%). When the reaction was repeated and stirred for 1 hour, a similar yield (20%) was obtained. $[\alpha]^{20}_D$ = -22.7 (c = 0.93, MeOH). IR (υ_{max}, film): 2959, 2944, 2887, 2438, 2348, 1735, 1716, 1516, 1475, 1366, 1164. ^1H (500 MHz, $CDCl_3$): δ = 0.89 (3H, t, *J* = 7.1, CH_2<u>C</u>H_3), 1.27-1.36 (4H, m, <u>C</u>H_2<u>C</u>$H_2$$CH_3$), 1.44 (9H, s, Boc), 1.56-1.64 (1H, m, Hβ), 1.74-1.82 (1H, m, Hβ′), 3.73 (3H, s, CO_2<u>Me</u>), 4.28 (1H, m, Hα), 4.99 (1H, d, *J* = 6.6, NH). ^{13}C (125 MHz, $CDCl_3$): δ = 13.8 (CH_2<u>C</u>H_3), 22.3, 27.4 (<u>C</u>H_2<u>C</u>$H_2$$CH_3$), 28.3 (Boc), 32.5 (Cβ), 52.2 (CO_2<u>Me</u>), 53.4 (Cα), 79.8 (Boc), 155.4, 173.5 (2 × C=O). HRMS m/z (ESI^+): Found 268.1519 $[M+Na]^+$; $C_{12}H_{23}NO_4Na$ requires 268.1519.



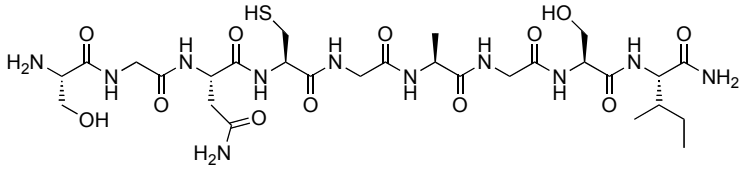
Phenylalanine methyl ester hydrochloride (100 mg, 0.46 mmol) was added to a 25 mL round bottom flask and suspended in DMF (5 mL). K_2CO_3 (384 mg, 2.78 mmol) was added and the mixture was stirred 5 minutes at room temperature before MSH (247 mg, 1.15 mmol) was added as a solid. The reaction was stirred for 10 minutes at room temperature and then diluted with Et_2O

(150 mL) and washed with $NaHCO_3$ (sat. aq., 100 mL) and brine (100 mL). The organic layer was dried ($MgSO_4$), filtered, and concentrated under reduced pressure. TLC (20% EtOAc in petrol) revealed a major product spot (R_f = 0.4). This product was purified by column chromatography (20% EtOAc in petrol) and identified as the deamination product **15** (36 mg, 47%). Analytical data of this material was consistent with that previously reported.[19] IR (υ_{max}, film): 3064, 3003, 2360, 2341, 1738, 1604, 1496, 1454, 1436, 1364, 1294, 1256, 1196, 1164. ^1H (500 MHz, $CDCl_3$): δ = 2.66 (2H, t, *J* = 7.9), 2.98 (2H, t, *J* = 7.9) ($C\underline{H_2}C\underline{H_2}$), 3.69 (3H, s, $CO_2\underline{Me}$), 7.20-7.25 (3H, m, ArH), 7.28-7.33 (2H, m, ArH). ^{13}C (125 MHz, $CDCl_3$): δ = 30.9, 35.7 ($\underline{C}H_2\underline{C}H_2$), 51.6 ($CO_2\underline{Me}$), 126.2, 128.2, 128.5 ($CH_{Ar}$), 140.5 ($4^o{}_{Ar}$), 173.3 (C=O). LRMS m/z (ESI^+): 187.1 $[M+Na]^+$.

MSH reaction on model peptide 16

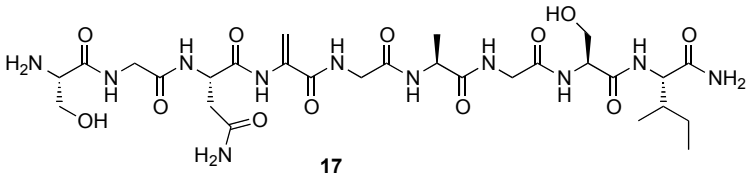
The peptides were isolated on preparative scale using reverse phase HPLC (Dionex Ultimate 3000) on a C18 column (Synergi 4u Fusion-RP 80A, 100 × 21.20 mm) with gradients of water containing 0.1% trifluoroacetic acid (solvent A) and acetonitrile containing 0.1% trifluoroacetic acid (solvent B). UV traces were detected for 204 and 214 nm. Peptides was analyzed by electrospray ionization mass spectrometry and purity was determined using analytical HPLC on a C18 column (Synergi 4u Fusion-RP 80A, 100 × 4.6mm)

Ser-Gly-Asn-Cys-Gly-Ala-Gly-Ser-Ile-NH_2 (16)



SBL peptide fragment with *C*-terminal amide (153-161); Ser-Gly-Asn-Cys-Gly-Ala-Gly-Ser-IleNH_2) was synthesized using Fmoc-chemistry on Rink amide MBHA-polystyrene resin [1% divinyl benzene, GL Biochem] using a microwave assisted Liberty CEM peptide synthesizer. Following cleavage from the resin [TFA/TIS/H_2O/EDT (94:1:2.5:2.5)], the peptide was purified by preparative RP-HPLC using the following gradient at a flow of 12 mL / min: 0-3 min 5% B; 3-15 min linear gradient 5% to 20% B; 15-18 min 20% B; 18-20 min linear gradient 20% to 90% B; 20-22 min 90% B; 22-24 min linear gradient 90% to 95% B; 24-26 min 95% B. HRMS (ESI^+) for (M+H) $C_{28}H_{50}N_{11}O_{12}S$ (*m/z*): calc. 764.3356; found 764.3354.

Ser-Gly-Asn-Dha-Gly-Ala-Gly-Ser-Ile-NH_2 (17)

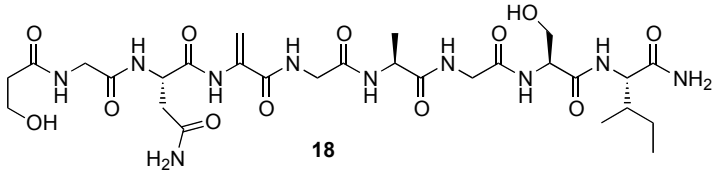


17

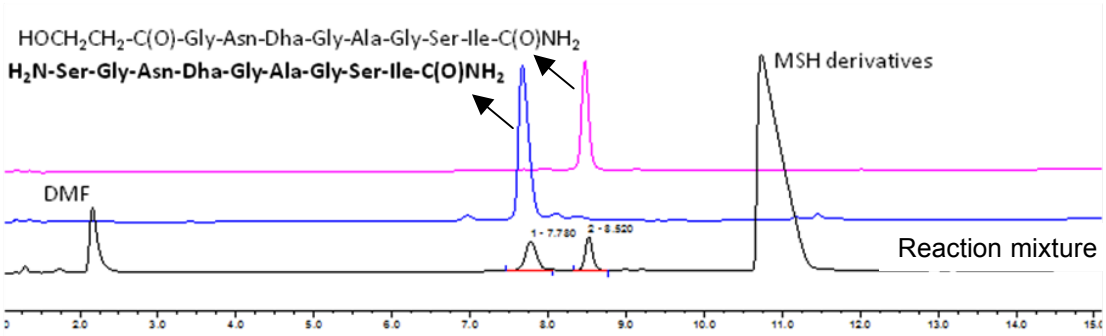
In a 25 mL round bottom flask, MSH (13.9 mg, 0.064 mmol) was dissolved in DMF (0.65 mL). In a separate vial, peptide **16** (8.0 mg, 0.010 mmol) was dissolved in deoxygenated H_2O (2.5 mL) and sodium carbonate was added (11 mg, 0.104 mmol). The resulting solution was added by pipette over a period of 3 min to the stirred MSH solution at room temperature. The vial was washed with 0.5 mL of H_2O. The reaction was stirred for additional 9 min, and quenched with TFA (pH ~4). The reaction mixture was purified by RP-HPLC and the collected fractions were lyophilized to provide peptide **17** (4.2 mg, 55%) and peptide **18** (3.1 mg, 41%) as white solids. HPLC traces are shown below.

HRMS (ESI^+) for **17** (M+H) $C_{28}H_{48}N_{11}O_{12}$ (*m/z*): calc. 730.3478; found 730.3473;

HRMS (ESI^+) for **18** (M+Na) $C_{28}H_{46}N_{10}O_{12}Na$ (*m/z*): calc. 737.3189; found 737.3200.



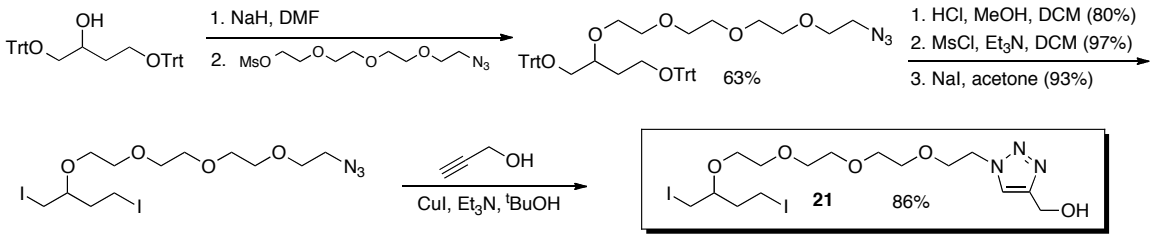
18

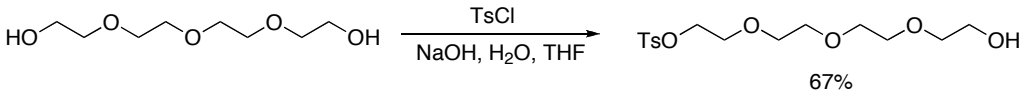


Analytical HPLC analysis of SGNDhaGAGSINH_2 **17** and deamination product **18**.

Bis-Alkylation-Elimination in the Conversion of Cysteine to Dehydroalanine

Synthesis of diiodide 21



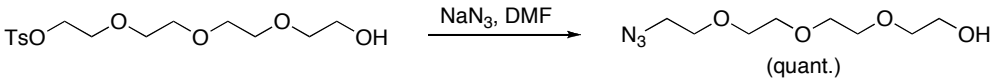


Tetraethylene glycol (20.0 g, 103 mmol) was added to a 250 mL round bottom flask and dissolved in THF (20 mL). The stirred solution was cooled to 0 ºC and then NaOH (1.32 g, 33.0 mmol) was added as a solution in H_2O (20 mL). Tosyl chloride (3.93 g, 20.6 mmol) was prepared as a solution in THF (20 mL) and then poured into the reaction flask. The reaction was stirred at 0 ºC, open to air, for 2 hours. After this time, the reaction mixture was diluted with EtOAc (200 mL) and H_2O (200 mL). The organic layer was separated, washed with brine (200 mL), dried ($MgSO_4$), filtered, and concentrated under reduced pressure. The resulting residue was purified by column chromatography (5% MeOH in EtOAc) to provide the tosylate as a clear oil (4.82 g, 67%). Spectroscopic data was consistent with that previously reported.[20] IR (υ_{max}, film): 3447, 2874, 1597, 1454, 1355, 1177, 1098, 1017, 924. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.41 (3H, s, CH_3), 3.55-3.68 (14H, m) 4.13 (2H, t, J = 4.8) (-OC$\underline{H_2}$C$\underline{H_2}$O-), 7.31 (2H, d, J = 8.1, ArH), 7.76 (2H, d, J = 8.1, ArH). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 21.5 (CH_3), 61.5, 68.5, 69.1, 70.1, 70.3, 70.47, 70.54, 72.3 (-O$\underline{C}H_2\underline{C}H_2$O-), 127.8, 129.7 ($\underline{C}H_{Ar}$), 132.7, 144.7 ($4^o{}_{Ar}$). LRMS (ESI+): 371.1 $(M+Na)^+$.

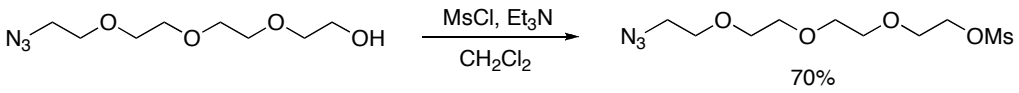
This reaction can also be done on a larger scale and no purification is required if excess tetraethylene glycol is used:

Tetraethylene glycol (200.0 g, 1.03 mol) was added to a 1 L round bottom flask and dissolved in THF (200 mL). The stirred solution was cooled to 0 ºC and NaOH (6.18 g, 154 mmol) was added as a solution in H_2O (100 mL). The solution was stirred at 0 ºC for 10 minutes before tosyl

chloride (24.54 g, 128.7 mmol) was added as a solution in THF (50 mL). The reaction was stirred for 2 hours at 0 ºC, open to air. After this time, the reaction was diluted with EtOAc (500 mL) and H_2O (500 mL). The organic layer was separated, washed with brine (500 mL), dried ($MgSO_4$), and filtered. Concentrating under reduced pressure provided the tosylate as a clear oil spectroscopically identical to the purified sample prepared above (32.92 g, 73%). This material was used in the next step without further purification.

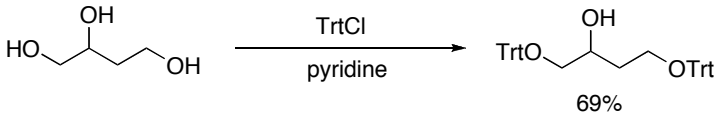


The tosylate prepared above (32.91 g, 94.46 mmol) was dissolved in DMF (50 mL). Sodium azide (18.42 g, 283.4 mmol) was added to the stirred solution and the reaction was heated to 90 ºC. The reaction was stirred, open to air, for 15 minutes. After this time, an additional 20 mL DMF was added to help with solubility. After a total of 1 hour at 90 ºC, TLC (5% MeOH in EtOAc) revealed complete consumption of starting material. The reaction was then diluted with H_2O (500 mL) and extracted with CH_2Cl_2 (3 × 200 mL). The combined organic layers were dried ($MgSO_4$), filtered, and concentrated under reduced pressure. The product azide was isolated as a clear oil and did not require purification (20.71 g, quant.). Spectroscopic data was consistent with that previously reported.[20] IR (υ_{max}, film): 3444, 2871, 2107, 1645, 1454, 1349, 1287, 1125, 937. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 3.36 (2H, t, *J* = 4.8), 3.57 (2H, t, *J* = 4.3), 3.61-3.67 (10H, m), 3.69 (2H, t, *J* = 4.3). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 50.5, 61.5, 69.9, 70.2, 70.43, 70.49, 70.53, 72.4. LRMS (ESI+): 242.1 $(M+Na)^+$.

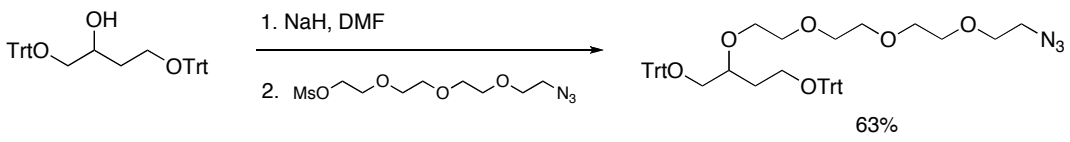


The azide derivative (20.71 g, 94.46 mmol) was added to a 500 mL round bottom flask and dissolved in CH_2Cl_2 (150 mL). Et_3N (40 mL, 283 mmol) was added and the stirred solution was cooled to 0 ºC. Methane sulfonylchloride (11.0 mL, 142 mmol) was added slowly and the reaction was stirred at 0 ºC for 10 min. The ice bath was then removed and the reaction stirred at room temperature for 1 hour. After this time, TLC (2% MeOH in EtOAc) revealed complete consumption of starting material. The reaction was diluted with CH_2Cl_2 (200 mL) and then washed with 1M HCl (200 mL). The aqueous layer was extracted with CH_2Cl_2 (200 mL). The

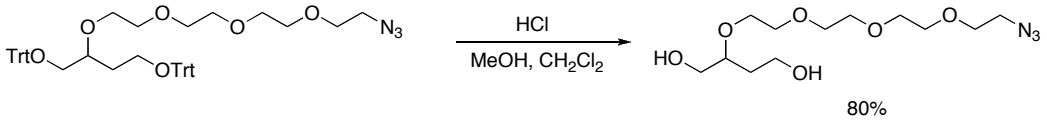
combined organic layers were dried ($MgSO_4$), filtered, and concentrated under reduced pressure. The resulting residue was then purified by column chromatography (2% MeOH in EtOAc) to afford the product mesylate as a yellow oil (19.73 g, 70%). Spectroscopic data was consistent with that previously reported.[21] IR (υ_{max}, film): 2872, 2109, 1454, 1414, 1174, 1128, 1017, 974, 922. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 3.05 (3H, s, $SO_2\underline{Me}$), 3.36 (2H, t, *J* = 5.1, $C\underline{H_2}N_3$), 3.63-3.65 (10H, m, -OCH_2CH_2O-), 3.74 (2H, t, *J* = 4.6, $C\underline{H_2}CH_2OMs$), 4.35 (2H, t, *J* = 4.6, $CH_2C\underline{H_2}OMs$). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 37.5 ($SO_2\underline{Me}$), 50.5 ($\underline{C}H_2N_3$), 68.8, 69.2, 69.9, 70.43 (2 × C), 70.47, 70.50 (-$O\underline{C}H_2\underline{C}H_2O$-). LRMS (ESI+): 320.1 $(M+Na)^+$.



This procedure was adapted from the literature.[22] 1,2,4-Butanetriol (1.00 g, 9.42 mmol) was added to a 100 mL round bottom flask and dried under high vacuum for 15 minutes. The flask was then placed under an atmosphere of nitrogen and the triol was dissolved in pyridine (25 mL). Triphenylmethyl chloride (6.30 g, 22.6 mmol) was added in one portion and the reaction stirred at room temperature for 20 hours. The reaction was then diluted with EtOAc (300 mL) and then washed with 1M HCl (2 × 150 mL). The organic layer was dried ($MgSO_4$), filtered, and concentrated under reduced pressure. The resulting residue was crystallized from CH_2Cl_2 and petrol. The product was isolated as small white crystals and dried under high vacuum (3.84 g, 69%). m.p. = 152-154 ºC. IR (υ_{max}, film): 3566, 3085, 2926, 2876, 2360, 1596, 1490, 1447, 1032, 762, 647, 633. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.83 (2H, q, *J* = 5.8, $C\underline{H_2}CH_2OTrt$), 2.86 (1H, app s, OH), 3.12-3.16 (1H, m), 3.18-3.23 (2H, m), 3.29-3.35 (1H, m) (2 × $C\underline{H_2}OTrt$), 4.07 (1H, br app s, C<u>H</u>OH), 7.27-7.34 (18H, m), 7.44-7.48 (12H, m) (2 × Trt). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 33.7 ($\underline{C}H_2CH_2OTrt$), 61.2 ($CH_2\underline{C}H_2OTrt$), 67.5 ($\underline{C}H_2OTrt$), 69.6 (<u>C</u>HOH), 86.5, 86.9 (2 × Trt), 126.9, 127.0, 127.8 (2 × C), 128.5, 128.6, 143.9, 144.0 (2 × Trt). LRMS (ESI+): 613.2 $(M+Na)^+$.

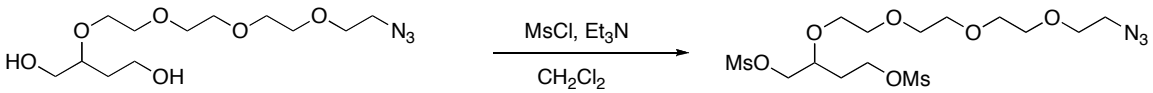


The mesylate (12.04 g, 40.49 mmol) was added to a 25 mL round bottom flask and dried under high vacuum for 30 minutes prior to use. The bis-trityl ether (15.95 g, 26.99 mmol) was added to a flame dried 2-neck, 250 mL round bottom flask and placed under an atmosphere of nitrogen before dissolving in anhydrous DMF (50 mL). Sodium hydride (1.62 g, 60% in mineral oil, 40.5 mmol) was added carefully at room temperature and an efficient exit for hydrogen gas was ensured. The reaction mixture was stirred for 10 minutes at room temperature before the dried mesylate was added as a solution in DMF (10 mL). After 3 hours at room temperature, the reaction was cooled to 0 ºC and quenched by the careful addition of saturated aqueous NH_4Cl (50 mL). The mixture was then diluted with EtOAc (300 mL) and washed with H_2O (300 mL) and brine (300 mL). The organic layer was then dried ($MgSO_4$), filtered, and concentrated under reduced pressure. The resulting residue was purified by column chromatography (25% EtOAc in petrol) to afford alkylated product as a thick, clear oil (13.49 g, 63%). IR (υ_{max}, film): 3085, 3058, 3031, 2872, 2104, 1596, 1490, 1448, 1285, 1182, 1089, 763, 747, 706, 632. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.75-1.87 (2H, m, C<u>H</u>$_2$CH$_2$OTrt), 3.09-3.13 (4H, m, 2 × C<u>H</u>$_2$OTrt), 3.34 (2H, t, *J* = 5.0, CH_2N_3), 3.45-3.50 (3H, m), 3.54-3.57 (4H, m), 3.60-3.70 (7H, m), 3.73-3.78 (1H, m) (-OC<u>H</u>$_2$C<u>H</u>$_2$O- and C<u>H</u>CH$_2$OTrt), 7.19-7.29 (18H, m), 7.38-7.45 (12H, m) (2 × Trt). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 32.7 (C<u>H</u>$_2$CH$_2$OTrt), 50.5 (CH_2N_3), 59.9, 66.0, 69.62, 69.89, 70.44, 70.52 (2 × C), 70.55, 70.66 (-OC<u>H</u>$_2$C<u>H</u>$_2$O- and 2 × C<u>H</u>$_2$OTrt), 76.8 (C<u>H</u>CH$_2$OTrt), 86.3, 86.4 (2 × Trt), 126.74, 126.79, 127.62, 127.67, 128.55, 128.65, 144.06, 144.24 (2 × Trt). HRMS m/z (ESI$^+$): Found 814.3832 $[M+Na]^+$; $C_{50}H_{53}N_3O_6Na$ requires 814.3827.

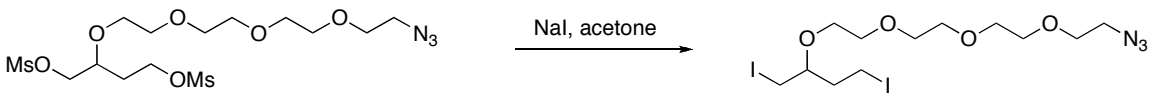


Anhydrous MeOH (50 mL) was added to a 250 mL round bottom flask and placed under an atmosphere of nitrogen. The flask was cooled to 0 ºC and acetyl chloride (12.2 mL, 170.3 mmol) was added slowly. The solution was stirred for 10 minutes to generate methanolic HCl. The Bis-trityl ether (13.49 g, 17.03 mmol) was added to the reaction flask as a solution in CH_2Cl_2 (30 mL). The reaction was then warmed to room temperature and stirred for 2 hours after which time TLC (5% MeOH in CH_2Cl_2) revealed complete consumption of starting material. Volatile components were removed under reduced pressure and the resulting residue was purified directly

by column chromatography (5% MeOH in CH_2Cl_2 then 7% MeOH in CH_2Cl_2). The product was isolated as a thick, clear oil (4.20 g, 80%). IR (υ_{max}, film): 3418, 2873, 2108, 1646, 1454, 1347, 1300, 1120, 945, 850. ^{1}H NMR (400 MHz, MeOD): δ = 1.72 (2H, q, *J* = 6.1, C<u>H</u>$_2$CH$_2$OH), 3.39 (2H, t, *J* = 4.9, CH_2N_3), 3.50-3.58 (2H, m), 3.61-3.72 (16H, m), 3.76-3.79 (1H, m) (-OC<u>H</u>$_2$C<u>H</u>$_2$O-, 2 × C<u>H</u>$_2$OH, and C<u>H</u>CH$_2$OH). ^{13}C NMR (100 MHz, MeOD): δ = 35.5 (<u>C</u>H$_2$CH$_2$OH), 51.9 (CH_2N_3), 59.8, 65.1, 70.36, 71.26, 71.65 (2 × C), 71.67, 71.74, 72.05 (-O<u>C</u>H$_2$<u>C</u>H$_2$O- and 2 × <u>C</u>H$_2$OH), 79.7 (<u>C</u>HCH$_2$OH). HRMS m/z (ESI$^+$): Found 330.1637 $[M+Na]^+$; $C_{12}H_{25}N_3O_6Na$ requires 330.1636.

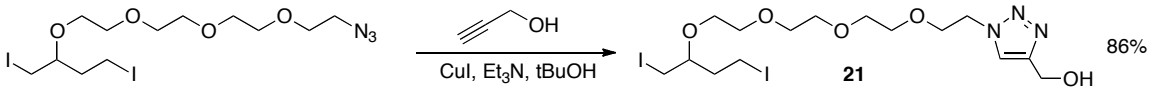


The diol (4.20 g, 13.7 mmol) was added to a 250 mL round bottom flask and dissolved in CH_2Cl_2 (60 mL). Et_3N (11.4 mL, 82.0 mmol) was added and the resulting solution was cooled to 0 ºC. Methane sulfonylchloride (3.17 mL, 41.0 mmol) was added slowly to the reaction. After the addition was complete, the reaction was stirred for 5 minutes before the ice bath was removed. The reaction was then stirred at room temperature for 1hour. After this time the reaction was quenched by diluting with CH_2Cl_2 (100 mL) and 1M HCl (100 mL). The organic layer was separated and the aqueous layer was extracted with CH_2Cl_2 (2 × 70 mL). The combined organic layers were dried ($MgSO_4$), filtered, and concentrated under reduced pressure. The resulting residue was purified by column chromatography (2% MeOH in CH_2Cl_2) to afford the busulfan derivative as a clear oil (6.13 g, 97%). IR (υ_{max}, film): 3022, 2937, 2874, 2109, 1463, 1352, 1174, 1123, 950, 836. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.92-1.99 (2H, m, C<u>H</u>$_2$CH$_2$OMs), 3.01 (3H, s), 3.05 (3H, s) (2 × SO_2Me), 3.36 (2H, t, *J* = 5.1, CH_2N_3), 3.58-3.66 (13H, m), 3.75-3.82 (2H, m) (-OC<u>H</u>$_2$C<u>H</u>$_2$O- and C<u>H</u>CH$_2$OMs), 4.16 (1H, dd, *J* = 11.1, 4.8), 4.29-4.43 (3H, m) (2 × C<u>H</u>$_2$OMs). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 31.1 (<u>C</u>H$_2$CH$_2$OMs), 37.1, 37.4 (2 × SO_2Me), 50.5 (CH_2N_3), 66.0, 69.8, 69.9, 70.27, 70.32, 70.37, 70.43, 70.44, 70.47 (-O<u>C</u>H$_2$<u>C</u>H$_2$O- and 2 × <u>C</u>H$_2$OMs), 73.7 (<u>C</u>HCH$_2$OMs). HRMS m/z (ESI$^+$): Found 486.1189 $[M+Na]^+$; $C_{14}H_{29}N_3O_{10}SNa$ requires 486.1187.



93%

The Busulfan derivative (2.00 g, 4.31 mmol) was added to a 100 mL round bottom flask and dissolved in acetone (25 mL). Sodium iodide (6.47 g, 43.2 mmol) was added and the flask was then equipped with a condenser and placed under an atmosphere of nitrogen. The reaction was heated to reflux and stirred for 5 hours. After this time, the reaction was cooled to room temperature and diluted with EtOAc (200 mL) and H_2O (200 mL). The organic layer was separated and then washed with brine (200 mL). The organic layer was dried ($MgSO_4$), filtered, and concentrated under reduced pressure. The resulting residue was purified by column chromatography (60% EtOAc in petrol) to afford the di-iodide as a clear oil (2.12 g, 93%). IR (υ_{max}, film): 2868, 2103, 1439, 1347, 1297, 1246, 1114. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.04-2.17 (2H, m, C$\underline{H}_2$CH_2I), 3.24-3.31 (4H, m, 2 × CH_2I), 3.37-3.42 (3H, m, C$\underline{H}_2$N$_3$ and C$\underline{H}$CH_2I), 3.59-3.68 (13H, m), 3.76-3.82 (1H, m) (-OCH_2CH_2O-). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 2.19, 8.59 (2 × CH_2I), 38.7 (C$\underline{H}_2$CH_2I), 50.6 (CH_2N_3), 69.25, 69.95, 70.59 (br, 3 × C), 70.63, 70.64 (-OCH_2CH_2O-), 78.3 (C$\underline{H}$CH_2I). HRMS m/z (ESI$^+$): Found 549.9647 [M+Na]$^+$; $C_{12}H_{23}N_3O_4I_2Na$ requires 549.9670.

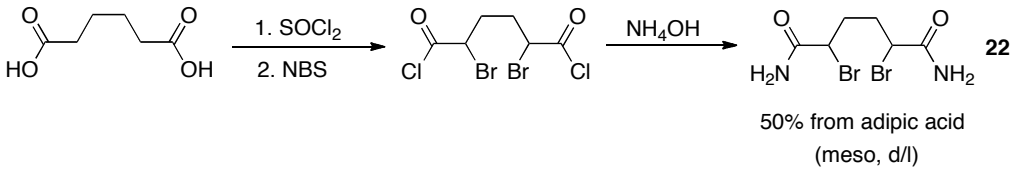


86%

The azide (149 mg, 0.28 mmol) was added to a 5 mL round bottom flask and dissolved in ^{t}BuOH (0.40 mL). Propargyl alcohol (33 μL, 0.56 mmol), Et_3N (0.20 mL, 1.4 mmol), and CuI (5 mg, 0.028 mmol) were added sequentially to the stirred solution. The flask was sealed with a septum and placed under an atmosphere of nitrogen. The reaction was stirred at room temperature for 3 hours after which time all starting material had been consumed (TLC, 5% MeOH in CH_2Cl_2). The reaction mixture was loaded directly to a silica column and purified by elution with 5% MeOH in CH_2Cl_2. Triazole **21** was isolated as a clear liquid (142 mg, 86%). IR (υ_{max}, film): 3385, 2869, 1600, 1456, 1351, 1181, 1096. ^{1}H NMR (400 MHz, DMSO): δ = 1.91-2.06 (2H, m, C$\underline{H}_2$CH_2I), 3.23-3.54 (3H, m, contains C$\underline{H}$CH_2I and CH_2C$\underline{H}_2$I), 3.37-3.46 (2H, m, CHC$\underline{H}_2$I), 3.48-3.57 (11H, m), 3.63-3.69 (1H, m) (-OC$\underline{H}_2$C$\underline{H}_2$O-), 3.81 (2H, t, *J* = 5.3, OC$\underline{H}_2$CH_2N), 4.48-4.51 (4H, m, includes C$\underline{H}_2$OH and OCH_2C$\underline{H}_2$N), 5.16 (1H, app s, OH), 7.93 (1H, s, CH_{Ar}). ^{13}C NMR (100 MHz, DMSO): δ = 3.5, 11.3 (2 × CH_2I), 38.7 (C$\underline{H}_2$CH_2I), 49.2, 55.1 (CH_2OH and OCH_2C$\underline{H}_2$N),

68.14, 68.80, 69.56, 69.67, 69.76 (br 2 × C), 69.84 (-O<u>C</u>H$_2$<u>C</u>H$_2$O-), 77.4 (<u>C</u>HCH$_2$I), 123.1 (<u>C</u>H$_{Ar}$), 147.9 (4°$_{Ar}$). HRMS m/z (ESI$^+$): Found 605.9938 [M+Na]$^+$; $C_{15}H_{27}N_3O_5I_2Na$ requires 605.9932.

Synthesis of di-bromide 22

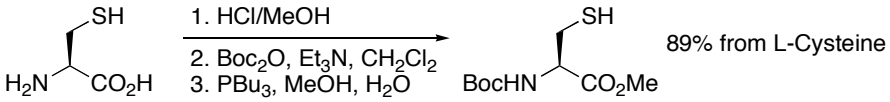


α,α′-Di-bromo-adipyl(bis)amide was prepared using a procedure adapted from the literature.[23, 24] Adipic acid (25.00 g, 171.1 mmol) was added to a 500 mL round bottom flask and suspended in thionylchloride (75.0 mL, 1034 mmol). The flask was equipped with a condenser and the reaction was heated to reflux (open to air, bath temp 80 ºC). After 30 minutes at reflux, all adipic acid had dissolved. The reaction was stirred for an additional 60 minutes at reflux and then cooled to room temperature. CCl_4 (100 mL) was added to the reaction followed by NBS (73.1 g, 411 mmol). The reaction was stirred vigorously and 10 drops of HBr (48% aq.) was added by pipette. The reaction was heated to reflux, again open to air. The reaction gradually turns from red to black over the course of an hour. After 2 hours at reflux, the reaction was cooled to room temperature and then to 0 ºC. The mixture was stirred at 0 ºC to ensure all succinimide had precipitated. The solid was removed by filtration. Et_2O (50 mL) was used to rinse and complete the filtration. The filtrate was concentrated under reduced pressure to give a thick, dark red liquid. In a 500 mL round bottom flask, 200 mL of NH_4OH (25% aq.) was cooled to 0 ºC. The crude acid chloride was added dropwise over 20 minutes to the ammonia solution with rapid stirring. After the addition was complete, the reaction was stirred vigorously at 0 ºC for 1 hour. The bis-amide product precipitated from the reaction mixture. The dark solid was isolated by filtration and partially dried. The product was purified by triturating in MeOH/H_2O: The solid was suspended in H_2O (100 mL) and MeOH (100 mL) and heated to 60 ºC. The mixture was stirred rapidly at 60 ºC for 30 minutes. After this time, the mixture was cooled to room temperature. The resulting white solid (a mixture of *meso* and *d/l* diastereomers) was isolated by filtration and washed with MeOH (200 mL). The product (**22**) was dried under high vacuum (25.75 g, 50%). IR (υ_{max}, KBr): 3302, 2946, 2801, 1684, 1418, 1319, 1277, 1250, 1220, 1194, 978, 876. ^{1}H NMR (400 MHz, DMSO): δ = 1.75-2.08 (4H, m, C<u>H$_2$</u>C<u>H$_2$</u>), 4.28-4.36 (2H, m, 2 × CHBr), 7.30 (2H, s), 7.69 (2H, s) (2 × NH_2).

^{13}C NMR (100 MHz, DMSO): (both diastereomers reported), δ = 32.5, 32.6 (CH_2CHBr), 48.2, 48.5 (CH_2CHBr), 169.87, 169.92 (C=O). LRMS m/z (ESI$^+$): 324.9 $[M+Na]^+$.

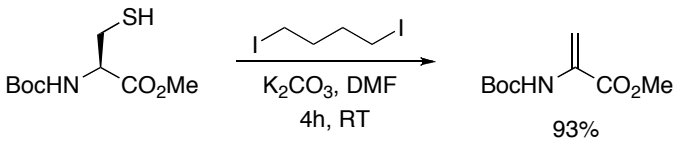
Di-alkylative conversion of cysteine to dehydroalanine:

BocCysOMe



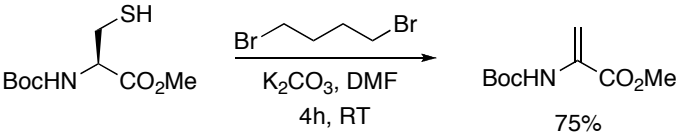
MeOH (100 mL) was added to a flame dried 250 mL round bottom flask under an atmosphere of argon. The solvent was stirred and cooled to 0 ºC and acetyl chloride (248 mmol, 17.6 mL) was added dropwise over 5 minutes. The solution was stirred an additional 10 minutes at 0 ºC to give a solution of methanolic HCl. L-cysteine (2.00 g, 16.5 mmol) was then added in one portion and the flask flushed with argon. The ice bath was removed and the reaction was stirred at room temperature for 24 hours under a balloon of argon. After this time, the solvent was then removed under reduced pressure to give the crude cysteine methyl ester hydrochloride as a pale yellow solid. This material was used immediately in the next step without purification. The crude ester was suspended in CH_2Cl_2 (100 mL) and cooled to 0 ºC. Et_3N (5.06 mL, 36.3 mmol) was added carefully followed by Boc_2O (4.32 g, 19.8 mmol). The reaction was stirred at room temperature for 3.25 hours after which time TLC (30% EtOAc in Petrol) revealed the desired product (R_f = 0.6) and its corresponding disulfide (R_f = 0.3). The solvent was removed under reduced pressure and the resulting residue was redissolved in MeOH (40 mL) and H_2O (8 mL). PBu_3 (2.0 mL, 8.1 mmol) was added dropwise to the stirred solution. TLC revealed reduction of the disulfide. The reaction was diluted with Et_2O (100 mL) and H_2O (50 mL). The organic layer was separated and the aqueous layer was extracted with Et_2O (2 × 50 mL). The combined organics were washed with brine (100 mL), dried over $MgSO_4$, and filtered. The solvent was removed by rotary evaporation and the residue purified by column chromatography eluting first with 5% EtOAc in petrol and then 20% EtOAc in petrol. BocCysOMe was isolated as a clear oil (3.48 g, 89% from L-cysteine). $[\alpha]^{20}_D$ = +28.3 (c = 7.5, $CHCl_3$) (Lit.[25]: $[\alpha]^{20}_D$ = +28.5, c = 7.5, $CHCl_3$). IR (υ_{max}, film): 2979, 1717, 1506, 1367, 1166. ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 1.42 (10H, s, Boc and SH), 2.94 (2H, app. td, *J* = 8.7, 4.3, CH_2SH), 3.76 (3H, s, CO_2Me), 4.58 (1H, m, Hα), 5.44 (1H, d, *J* = 5.8, NH). ^{13}C NMR (100 MHz): δ = 27.3 (CH_2SH), 28.2 (Boc), 52.6 (CO_2Me), 54.8 (Cα), 80.2 (Boc), 155.1, 170.8 (2 × C=O). LRMS (ESI$^-$): 234 $(M-H)^-$.

BocDhaOMe using 1,4-diiodobutane



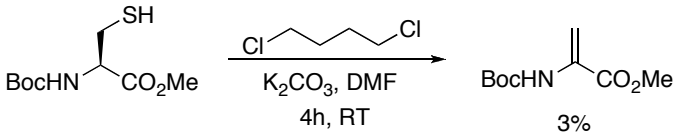
BocCysOMe (50 mg, 0.21 mmol) was added to a 25 mL round bottom flask and dissolved in DMF (5 mL). K_2CO_3 (147 mg, 1.06 mmol) was added to the stirred solution followed immediately by 1,4-diiodobutane (42 μL, 0.32 mmol). The flask was stoppered and the reaction stirred at room temperature for 4 hours. After this time, the reaction was diluted with Et_2O (100 mL) and washed sequentially with H_2O (100 mL) and brine (100 mL). The organic layer was dried ($MgSO_4$), filtered, and concentrated under reduced pressure. The resulting residue was purified by column chromatography (10% EtOAc in petrol) to provide BocDhaOMe as a clear oil (40 mg, 93%). IR (υ_{max}, film): 3423, 2980, 1719, 1634, 1513, 1328, 1159, 1068. ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 1.46 (9H, s, Boc), 3.80 (3H, s, $CO_2C\underline{H}_3$), 5.70 (1H, d, *J* = 1.5, C=C<u>H</u>H), 6.13 (1H, app s, C=CH<u>H</u>), 7.00 (1H, br s, NH). ^{13}C NMR (100 MHz): δ = 28.2 (Boc), 52.8 ($CO_2\underline{C}H_3$), 80.6 (Boc), 105.1 (C=$\underline{C}H_2$), 131.3 ($\underline{C}$=CH_2), 152.5, 164.4 (2 × C=O). Anal. for $C_9H_{15}NO_4$: C, 53.72; H, 7.51; N, 6.96; found C, 53.95; H, 7.63; N, 6.83.

BocDhaOMe using 1,4-dibromobutane



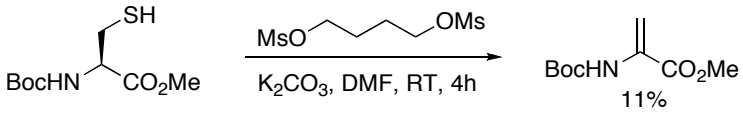
BocCysOMe (50 mg, 0.21 mmol) was added to a 25 mL round bottom flask and dissolved in DMF (5 mL). K_2CO_3 (147 mg, 1.06 mmol) was added to the stirred solution followed immediately by 1,4-dibromobutane (38 μL, 0.32 mmol). The flask was stoppered and the reaction stirred at room temperature for 4 hours. After this time, the reaction was diluted with Et_2O (100 mL) and washed sequentially with H_2O (100 mL) and brine (100 mL). The organic layer was dried ($MgSO_4$), filtered, and concentrated under reduced pressure. The resulting residue was purified by column chromatography (10% EtOAc in petrol) to provide BocDhaOMe as a clear oil (32 mg, 75%). Spectroscopic data was identical to that reported above.

BocDhaOMe using 1,4-dichlorobutane



BocCysOMe (50 mg, 0.21 mmol) was added to a 25 mL round bottom flask and dissolved in DMF (5 mL). K_2CO_3 (147 mg, 1.06 mmol) was added to the stirred solution followed immediately by 1,4-dichlorobutane (36 μL, 0.32 mmol). The flask was stoppered and the reaction stirred at room temperature for 4 hours. After this time, the reaction was diluted with Et_2O (100 mL) and washed sequentially with H_2O (100 mL) and brine (100 mL). The organic layer was dried ($MgSO_4$), filtered, and concentrated under reduced pressure. The resulting residue was purified by column chromatography (10% EtOAc in petrol) to provide BocDhaOMe as a clear oil (1.4 mg, 3%). Spectroscopic data was identical to that reported above.

BocDhaOMe using Busulfan



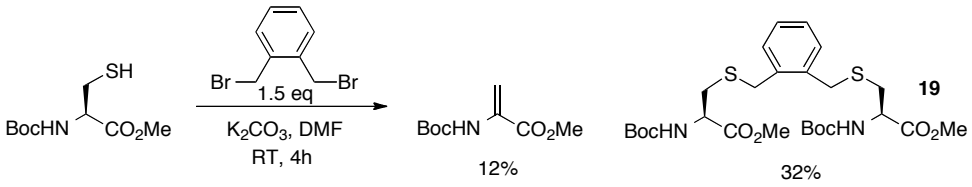
BocCysOMe (55 mg, 0.23 mmol) was added to a 50 mL round bottom flask and dissolved in DMF (5 mL). K_2CO_3 (162 mg, 1.17 mmol) was added to the stirred solution, followed immediately by Busulfan (85 mg, 0.35 mmol). The flask was stoppered and the reaction was stirred at room temperature for 4 hours. After this time, the reaction was diluted with Et_2O (100 mL) and washed sequentially with H_2O (100 mL) and brine (100 mL). The organic layer was dried ($MgSO_4$), filtered, and concentrated under reduced pressure. The resulting residue was purified by column chromatography (10% EtOAc in petrol) to provide BocDhaOMe (5 mg, 11%). Spectroscopic data was identical to that reported above.

BocDhaOMe using 1,5-diiodopentane

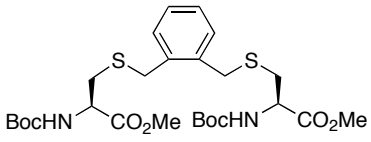
BocCysOMe (50 mg, 0.21 mmol) was added to a 25 mL round bottom flask and dissolved in DMF (5 mL). K_2CO_3 (147 mg, 1.06 mmol) was added followed immediately by 1,5-

diiodopentane (47 μL, 0.32 mmol). The reaction was stoppered and stirred at room temperature for 4 hours. After this time, the reaction was diluted with Et_2O (100 mL) and washed sequentially with H_2O (100 mL) and brine (100 mL). The organic layer was dried ($MgSO_4$), filtered, and concentrated under reduced pressure. The resulting residue was purified by column chromatography (10% EtOAc in petrol) to provide BocDhaOMe (5 mg, 12%). Spectroscopic data was identical to that reported above.

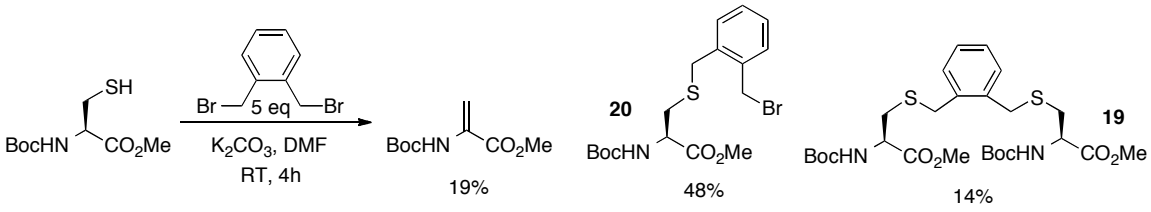
BocDhaOMe using α,α′-dibromo-*o*-xylene



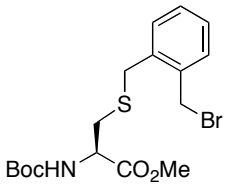
BocCysOMe (50 mg, 0.21 mmol) was added to a 50 mL round bottom flask and dissolved in DMF (5 mL). K_2CO_3 (147 mg, 1.06 mmol) was added, followed immediately by α,α′-dibromo-*o*-xylene (84 mg, 0.32 mmol). The reaction was stoppered and stirred at room temperature for 4 hours. After this time, the reaction was diluted with Et_2O (100 mL) and washed sequentially with H_2O (100 mL) and brine (100 mL). The organic layer was dried ($MgSO_4$), filtered, and concentrated under reduced pressure. The resulting residue was purified by column chromatography by first eluting with 10% EtOAc in petrol to provide BocDhaOMe (5 mg, 12%) and then with 40% EtOAc in petrol to provide **19** (19 mg, 32%). Spectroscopic data for BocDhaOMe was identical to that described above.



Data for **19**: m.p. = 130-131 ºC (MeOH). $[\alpha]^{20}_D$ = -3.7 (c = 0.8, $CHCl_3$). IR (υ_{max}, film): 3366, 2977, 1746, 1715, 1505, 1392, 1249, 1214, 1166, 1053, 1016. ^{1}H NMR ($CDCl_3$, 500MHz): δ = 1.46 (18H, s, Boc), 2.86-2.98 (4H, ABX system, *J* = 13.9, 5.4, 4.7, Hβ $_{Cys}$), 3.76 (6H, s, CO_2Me), 3.84-3.94 (4H, AB System, *J* = 12.9, S$\underline{C}H_2$Ar), 4.57 (2H, m, Hα $_{Cys}$), 5.39 (2H, d, *J* = 7.9, NH), 7.21-7.29 (4H, m, CH_{Ar}). ^{13}C NMR (125 MHz, $CDCl_3$): δ = 28.3 (Boc), 33.9, 34.2 (Cβ $_{Cys}$ and S$\underline{C}H_2$Ar), 52.6 (CO_2Me), 53.2 (Cα $_{Cys}$), 80.2 (Boc), 127.7, 130.9, 135.7 (Ar), 155.1, 171.6 (2 × C=O). HRMS m/z (ESI$^+$): Found 595.2125 $[M+Na]^+$; $C_{26}H_{40}N_2O_8S_2Na$ requires 595.2118.

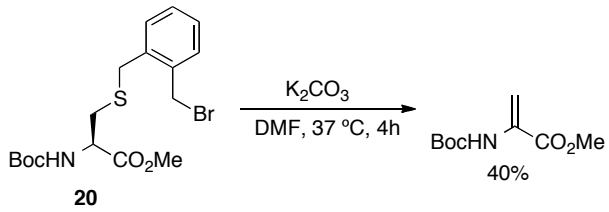


BocCysOMe (50 mg, 0.21 mmol) was added to a 50 mL round bottom flask and dissolved in DMF (3 mL). K_2CO_3 (147 mg, 1.06 mmol) was added followed immediately by a solution of α,α′-dibromo-*o*-xylene (280 mg, 1.06 mmol) in DMF (1 mL). An additional 1 mL of DMF was used to rinse and ensure complete transfer. The reaction was stoppered and stirred at room temperature for 4 hours. After this time, the reaction was diluted with Et_2O (100 mL) and washed sequentially with H_2O (100 mL) and brine (100 mL). The organic layer was dried ($MgSO_4$), filtered, and concentrated under reduced pressure. The resulting residue was purified by column chromatography by first eluting with 10% EtOAc in petrol to provide BocDhaOMe (8 mg, 19%) and then with 40% EtOAc in petrol to provide uncyclized **20** (41 mg, 48%) and **19** (8 mg, 14%). Spectroscopic data for BocDhaOMe and **19** was identical to that described above.



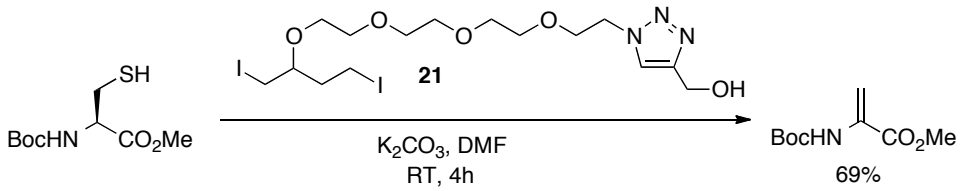
Data for uncyclized product **20**: clear oil. $[\alpha]^{20}_D$ = -5.3 (c = 1.7, $CHCl_3$). IR (υ_{max}, film): 3376, 2977, 2360, 2341, 1746, 1714, 1498, 1366, 1165. ^{1}H NMR ($CDCl_3$, 400MHz): δ = 1.46 (9H, s, Boc), 2.87-3.02 (2H, ABX system, *J* = 13.9, 5.6, 4.6, Hβ $_{Cys}$), 3.77 (CO_2Me), 3.92 (2H, AB system, *J* = 12.9, S$\underline{C}H_2$Ar), 4.58 (1H, m, Hα $_{Cys}$), 4.67 (2H, AB system, *J* = 10.4, CH_2Br), 5.35 (1H, d, *J* = 7.1, NH), 7.25-7.36 (4H, m, CH_{Ar}). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 28.3 (Boc), 30.9 (CH_2Br), 33.8, 34.5 (Cβ $_{Cys}$ and S$\underline{C}H_2$Ar), 52.6 (CO_2Me), 53.2 (Cα $_{Cys}$), 80.2 (Boc), 128.1, 129.0, 130.9, 131.1 (CH_{Ar}), 136.16, 136.22 (4° $_{Ar}$), 155.1, 171.5 (2 × C=O). HRMS m/z (ESI$^+$): Found 440.0502 $[M+Na]^+$; $C_{17}H_{24}NO_4SBrNa$ requires 440.0502.

Conversion of 20 to BocDhaOMe



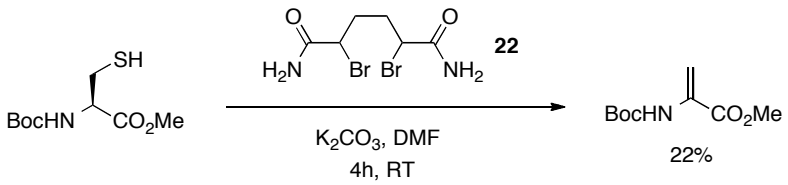
Uncyclized cysteine derivative **20** (236 mg, 0.560 mmol) was added to a 50 mL round bottom flask and dissolved in DMF (6 mL). K_2CO_3 (390 mg, 2.82 mmol) was added to the stirred solution and the reaction was heated to 37 ºC. After 4 hours, the reaction was diluted with Et_2O (150 mL) and then washed with H_2O (100 mL) and brine (100 mL). The organic layer was dried ($MgSO_4$), filtered, and concentrated under reduced pressure. The resulting residue was purified by column chromatography (10% EtOAc in petrol) to provide BocDhaOMe as a clear oil (45 mg, 40%). Spectroscopic data was identical to that reported above.

BocDhaOMe using 21

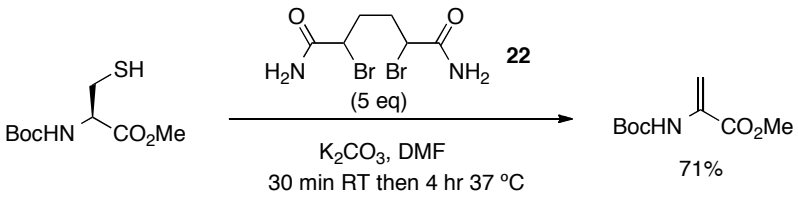


BocCysOMe (34 mg, 0.14 mmol) was added to a 25 mL round bottom flask and dissolved in DMF (2 mL). K_2CO_3 (97 mg, 0.70 mmol) was added followed immediately by a solution of triazole **21** (126 mg, 0.220 mmol) in DMF (1.5 mL). An additional 1.5 mL DMF was used to rinse and complete the transfer. The flask was stoppered and the reaction stirred at room temperature for 4 hours. After this time, the reaction was diluted with Et_2O (100 mL) and washed sequentially with H_2O (100 mL) and brine (100 mL). The organic layer was dried ($MgSO_4$), filtered, and concentrated under reduced pressure. The resulting residue was purified by column chromatography (10% EtOAc in petrol) to provide BocDhaOMe (20 mg, 69%). Spectroscopic data was identical to that reported above.

BocDhaOMe using 22

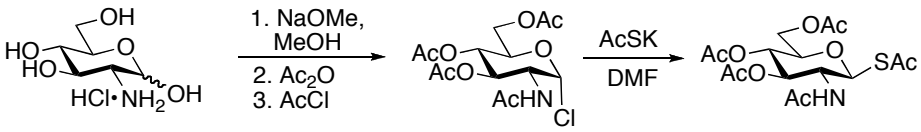


BocCysOMe (100 mg, 0.42 mmol) was added to a 10 mL round bottom flask and dissolved in DMF (3 mL). K_2CO_3 (290 mg, 2.10 mmol) was added to the stirred solution followed immediately by a solution of di-bromide **22** (192 mg, 0.640 mmol) in DMF (2 mL). An additional 1 mL of DMF was used to wash and complete the transfer. The flask was stoppered and the reaction stirred at room temperature for 4 hours. After this time, the reaction was diluted with Et_2O (80 mL) and washed sequentially with H_2O (80 mL) and brine (80 mL). The organic layer was dried ($MgSO_4$), filtered, and concentrated under reduced pressure. The resulting residue was purified by column chromatography (10% EtOAc in petrol) to provide BocDhaOMe as a clear oil (19 mg, 22%). Spectroscopic data was identical to that reported above.



BocCysOMe (459 mg, 1.95 mmol) was added to a 50 mL round bottom flask and dissolved in 20 mL DMF. K_2CO_3 (1.35 g, 9.77 mmol) and di-bromide **22** (2.95 g, 9.77 mmol) were both added as solids. The reaction was stirred at room temperature for 30 minutes and then placed in a water bath pre-heated to 37 ºC. After stirring for 4 hours at 37 ºC, the reaction was diluted with EtOAc (250 mL) and then washed with H_2O (150 mL) and brine (150 mL). The organic layer was dried ($MgSO_4$), filtered, and concentrated under reduced pressure. The resulting residue was purified by column chromatography (10% EtOAc in petrol) to afford BocDhaOMe as a clear oil (279 mg, 71%). Spectroscopic data was identical to that reported above.

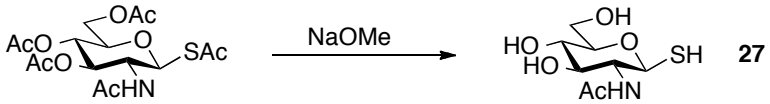
3,4,6-Tri-*O*-acetyl-*N*-acetyl-*S*-acetyl-1-thio-β-D-glucosamine



(D)-Glucosamine hydrochloride (20.0 g, 92.7 mmol) was added to a 100 mL round bottom flask and suspended in MeOH (20 mL). Sodium methoxide (6.00 g, 111 mmol) was added in several portions and the mixture stirred vigorously at room temperature for 1 hour before NaCl was removed by filtration. The solids were washed with MeOH (20 mL). Acetic anhydride (12 mL, 127 mmol) was added to the stirred filtrate at 0 ºC and warmed slowly to room temperature. After 12 hours, the reaction was concentrated to ~20 mL total volume and the product precipitated by the addition of Et_2O (100 mL). The solid was isolated by filtration and dried under vacuum before returning to an oven dried 2-neck 250 mL round bottom flask equipped with a condenser. The flask was placed under and argon atmosphere and cooled to 0 ºC. Acetyl chloride (40.0 mL, 563 mmol) was added slowly and the mixture stirred vigorously. After the addition, the reaction was heated to reflux. Gradually the reaction turned black. After 3 hours at reflux, the reaction was cooled to room temperature, diluted with CH_2Cl_2 (200 mL), and poured into a beaker of ice (~200 g). The organics were washed with $NaHCO_3$ (sat. aq., 2 × 200 mL), dried ($MgSO_4$), filtered, and concentrated to a black residue. The residue was dissolved in EtOAc and filtered through a short column of silica, eluting with EtOAc. The product was precipitated by the addition of cold Et_2O and petrol. The resulting powder was isolated by filtration and dried under vacuum (10.19 g, 50% from glucosamine hydrochloride). The crude product (~4:1 mixture of α:β) was used in the final step without purification. Accordingly, a portion of the anomeric chloride (1.00 g, 2.73 mmol) was added to a 50 mL round bottom flask and dissolved in DMF (15 mL). Potassium thioacetate (1.56 g, 13.7 mmol) was then added in one portion. The reaction was flushed with nitrogen and stirred at room temperature for 3 hours. After this time, no starting material was detected by TLC (70% EtOAc in petrol). The reaction mixture was diluted with EtOAc (250 mL) and washed sequentially with $NaHCO_3$ (sat. aq., 200 mL), H_2O (200 mL), and brine (200 mL). The organic layer was dried ($MgSO_4$), filtered, and concentrated under reduced pressure to give a brown solid. This solid was recrystallized from CH_2Cl_2 and petrol to afford the product as white crystals (661 mg, 60%). m.p. = 186-188 ºC (Lit[26]: 196-197, *i*PrOH). $[\alpha]^{20}_D$ = +12.1 (c = 2.2, $CHCl_3$) (Lit[26]: +14.1 c = 2.16, $CHCl_3$). IR (υ_{max}, KBr): 3733, 3064, 2961, 2360, 2341, 1747, 1707, 1665, 1538, 1433, 1370, 1230, 1048. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.90 (3H, s), 2.02 (6H, s), 2.05 (3H, s), 2.35 (3H, s) (5 × OAc), 3.81 (1H, ddd, *J* = 9.4, 4.6, 1.3, H5), 4.08 (1H, dd, *J* = 12.4, 1.3, H6),

4.22 (1H, dd, *J* = 12.4, 4.6, H6′), 4.34 (1H, q, *J* = 10.3, H2), 5.10-5.16 (2H, m, H3, H4), 5.17 (1H, d, *J* = 10.3, H1). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 20.49, 20.59, 20.64, 23.0, 30.8 (5 × OAc), 51.9 (C2), 61.8 (C6), 67.8, 74.0 (C3, C4), 76.3 (C5), 81.5 (C1), 169.2, 170.0, 170.6, 171.3, 193.5 (5 × C=O). LRMS (ESI+): 428.1 $(M+Na)^+$.

_N_-acetyl-1-thio-β-D-glucosamine (GlcNAc thiol 27)



27

97%

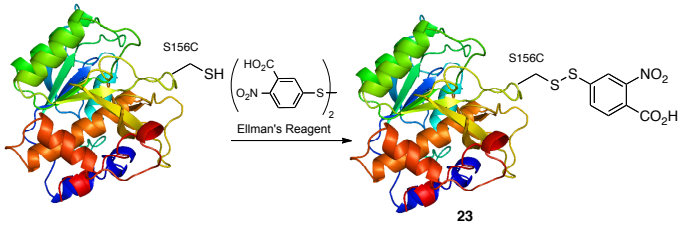
3,4,6-Tri-*O*-acetyl-*N*-acetyl-*S*-acetyl-1-thio-β-D-glucosamine (250 mg, 0.62 mmol) was added to a 50 mL round bottom flask and placed under an atmosphere of nitrogen before dissolving in anhydrous MeOH (8 mL). Sodium methoxide (67 mg, 1.2 mmol) was added under a stream of nitrogen at room temperature. The reaction was stirred for 1 hour at room temperature before quenching with DOWEX-50WX8 (H^+ form) until the pH of the reaction mixture was neutral (pH paper). The resin was removed by filtration and washed with MeOH (~ 50 mL). The filtrate was evaporated to provide GlcNAc thiol **27** as a thick syrup that crystallized upon standing (142 mg, 97%). m.p. = 170-171 ºC (Lit[27]: 174-176 ºC). $[\alpha]^{20}_D$ = -13.5 (c = 1.0, MeOH) (Lit[27]: -10.4, c = 1.0, MeOH). IR (υ_{max}, KBr): 3287, 2360, 2341, 1647, 1558, 1374, 1313, 1056. ^{1}H NMR (400 MHz, D_2O): δ = 1.90 (3H, s, Ac), 3.30-3.39 (3H, m, H3, H4, H5), 3.56 (1H, dd, *J* = 11.1, 5.3, H6), 3.59 (1H, t, *J* = 10.2, H2), 3.74 (1H, dd, *J* = 11.1, 1.2, H6′), 4.53 (1H, d, *J* = 10.2, H1). ^{13}C NMR (50 MHz, D_2O): δ = 22.6 (Ac), 58.2 (C2), 61.1 (C6), 70.0, 75.3, 79.3, 80.52 (C3, C4, C5, C1). LRMS (ESI-): 236.1 $(M-H)^-$. Note: in D_2O this material anomerizes to a 3.6:1.0 mixture of β:α over a period of 24 hours.

PROTEIN MODIFICATION

Protein Mass Spectrometry and Reaction Analysis

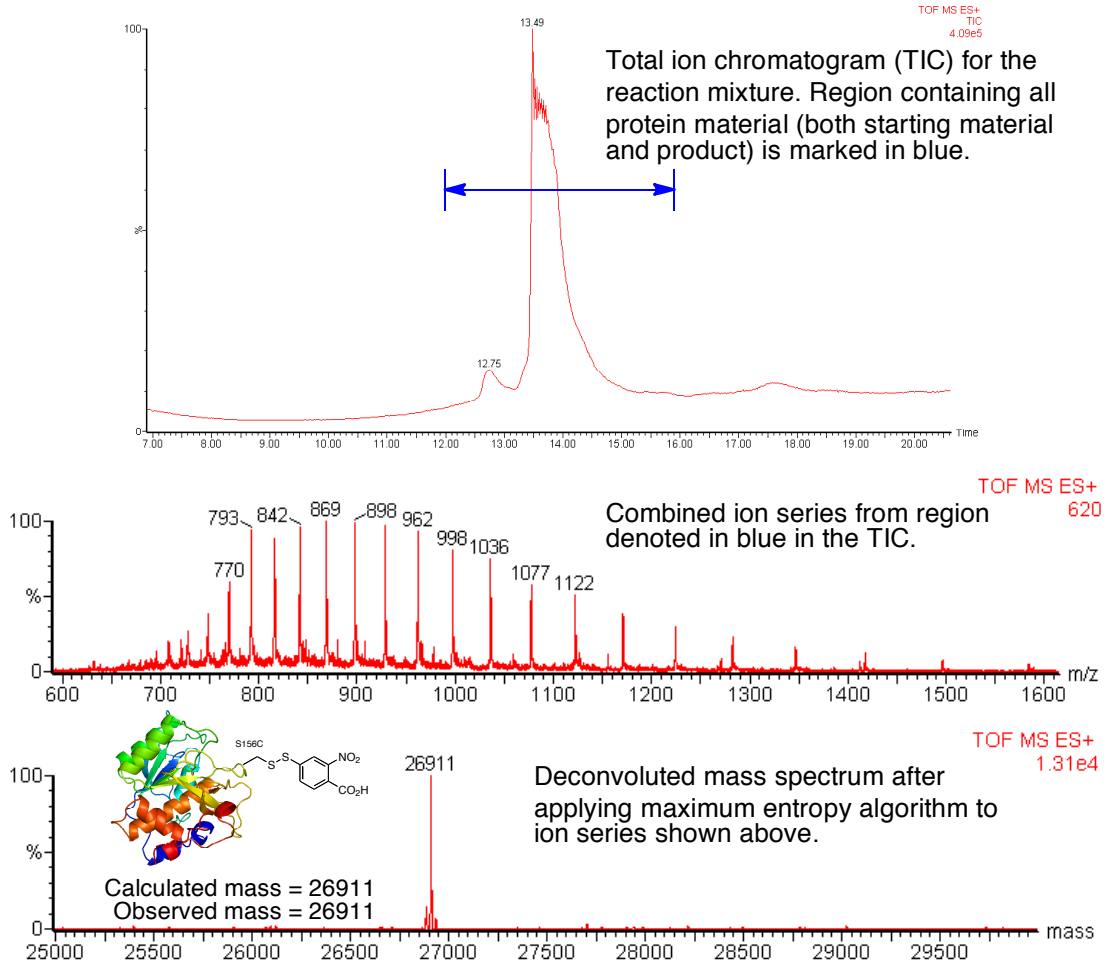
Liquid chromatography-mass spectrometry (LC-MS) was performed on a Micromass LCT (ESI-TOF-MS) coupled to an Agilent 1100 Series HPLC using a Phenomenex Jupiter 5 μm C4 column (250 × 4.6 mm). Water:acetonitrile, 95:5 (solvent A) and acetonitrile (solvent B), each containing 0.1% formic acid by volume, were used as the mobile phase at a flow rate of 1.0 mL/min. The gradient was programmed as follows: 95% A for 5 min to desalt and then a linear gradient to 100% B over 15 min followed by 100% B for an additional 5 min. A linear gradient over 10 minutes back to 95% A was used to re-equilibrate the column. The electrospray source was operated with a capillary voltage of 3.2 kV and a cone voltage of 25 V. Nitrogen was used as the nebulizer and desolvation gas at a total flow of 600 l/hr. Under these conditions, all protein material typically co-elutes in a single peak between 13 and 18 minutes. For reaction analysis, the mass spectra for all protein material in this peak were combined using MassLynx software (v. 4.0 from Waters). Mass spectra were then calibrated using a calibration curve constructed from a minimum of 17 matched peaks from the multiply charged ion series of equine myoglobin obtained at a cone voltage of 25V. The calibrated, combined ion series was deconvoluted using a maximum entropy algorithm that is preinstalled on the MassLynx software. If multiple proteins are present, the relative peak height is used to calculate the relative amount of each protein and reaction conversions. If a protein is modified at only a single site, we have shown previously that the relative MS peak height correlates well with the relative amount of protein measured by independent methods.[28] In the reactions considered in this report, excess reagents did not interfere with LC-MS analysis. In cases where reagents complicate MS analysis (for instance by co-eluting with protein material during LC-MS analysis) they can be removed by dialysis or size exclusion chromatography.[28]

For reference, a typical reaction analysis is described on the following page (the LC-MS analysis of the reaction between Ellman's reagent and SBL-S156C). The total ion chromatogram (TIC), combined ion series, and deconvoluted spectra are shown. A similar analysis was carried out for all protein reactions in this report.



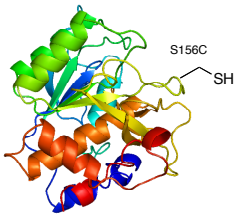
23

Representative LC-MS analysis for protein reactions:



Reduction-Elimination of Cysteine to Dehydroalanine: Attempts on SBL

SBL-S156C (PDB for wild type = 1GCI)



Sequence:

AQSVPWGISRVQAPAAHNRGLTGSGVKVAVLDTGISTHPDLNIRGGASFVPGEPSTQDG

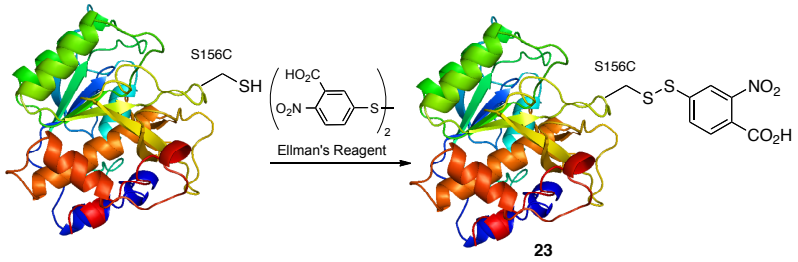
NGHGTHVAGTIAALNNSIGVLGVAPSAELYAVKVLGASGSGSVSSIAQGLEWAGNNGM

HVANLSLGSPSPSATLEQAVNSATSRGVLVVAASGNCGAGSISYPARYANAMAVGATD

QNNNRASFSQYGAGLDIVAPGVNVQSTYPGSTYASLNGTSMATPHVAGAAALVKQKNP

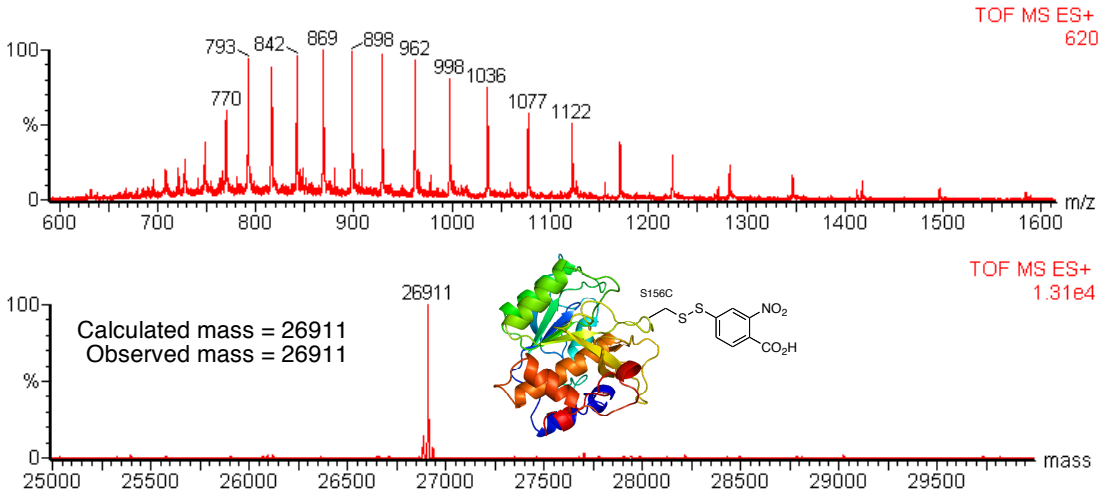
SWSNVQIRNHLKNTATSLGSTNLYGSGLVNAEAATR

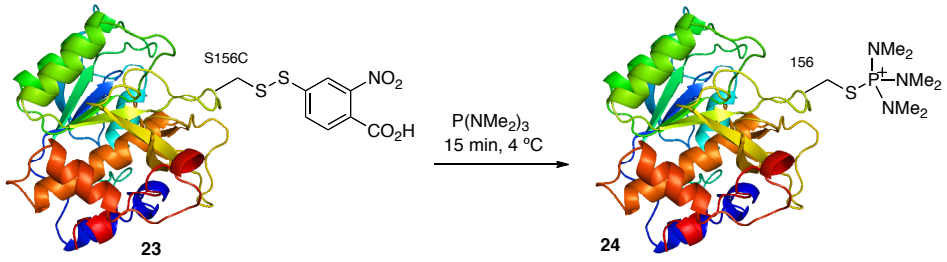
Average Mass = 26715



23

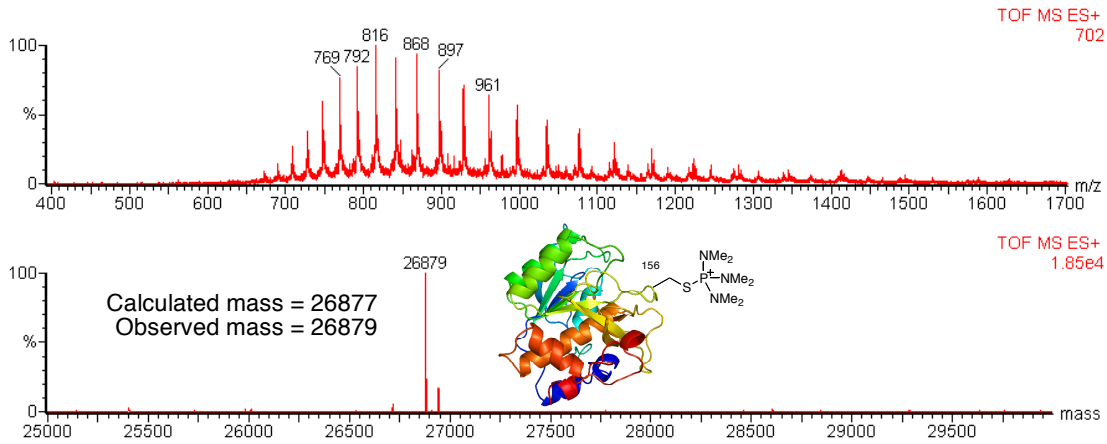
SBL-S156C was prepared as a 1 mg/mL solution in 50 mM potassium phosphate buffer (pH 8.0) and 200 µL were added to a 1.5 mL plastic tube. In a separate tube, Ellman's reagent (1.2 mg, 3.0 µmol) was dissolved in 200 µL of the same buffer. A 50 µL aliquot of the Ellman's solution was added to the protein and the reaction vortexed to homogenize. The resulting yellow solution was then shaken at 4 ºC for 20 minutes. LC-MS analysis revealed full conversion to the Ellman adduct **23** (calculated mass = 26911; observed mass = 26911).

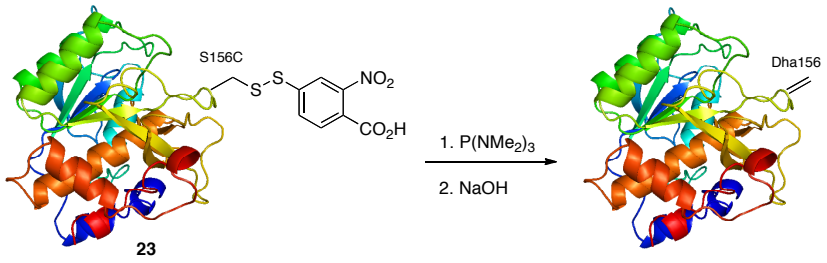




23 **24**

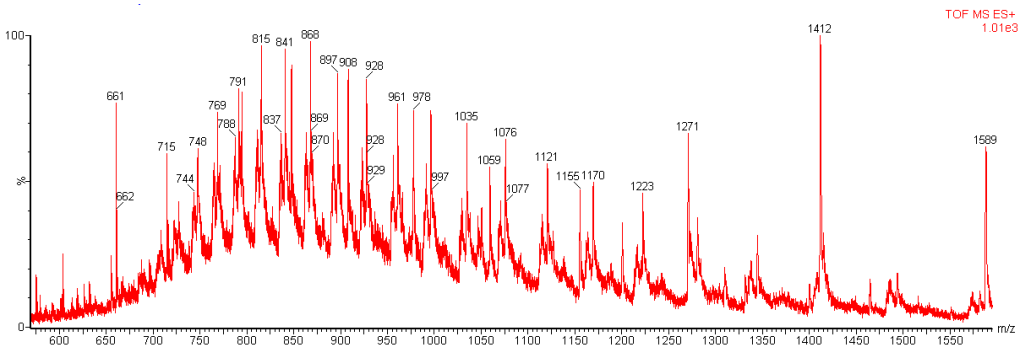
The SBL-S156C adduct of Ellman's reagent (**23**) was purified by dialysis twice against 4 L of 50 mM potassium phosphate (pH 8.0). HMPT (5 µL of a 0.06 mM solution in DMF, 0.3 µmol) was added to a 150 µL aliquot of the protein solution (0.3 mg/mL, 0.002 µmol). The reaction was shaken for 15 min at 4 ºC and then analyzed by LC-MS. The phosphonium salt **24** was observed as the major product (calculated mass = 26877; observed mass = 26879).

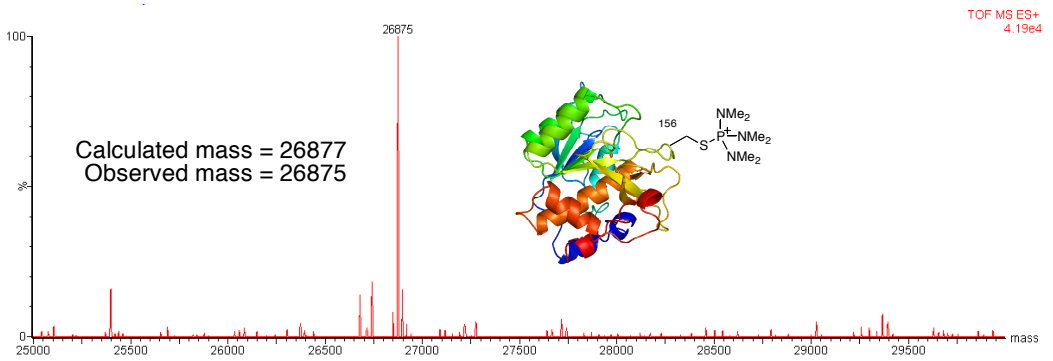


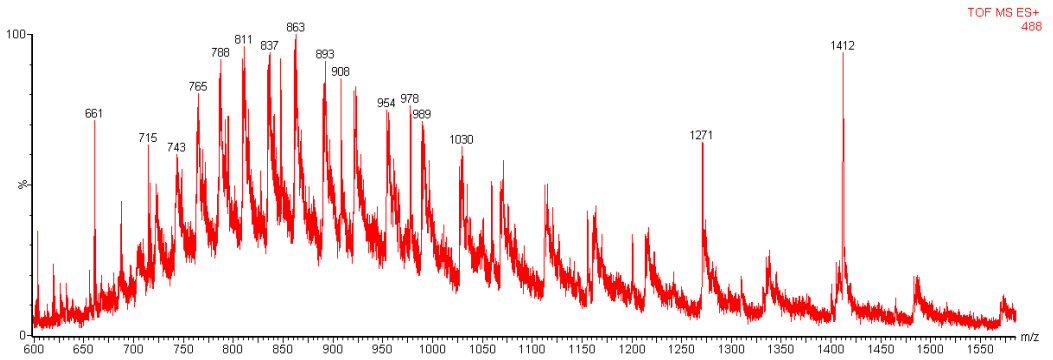


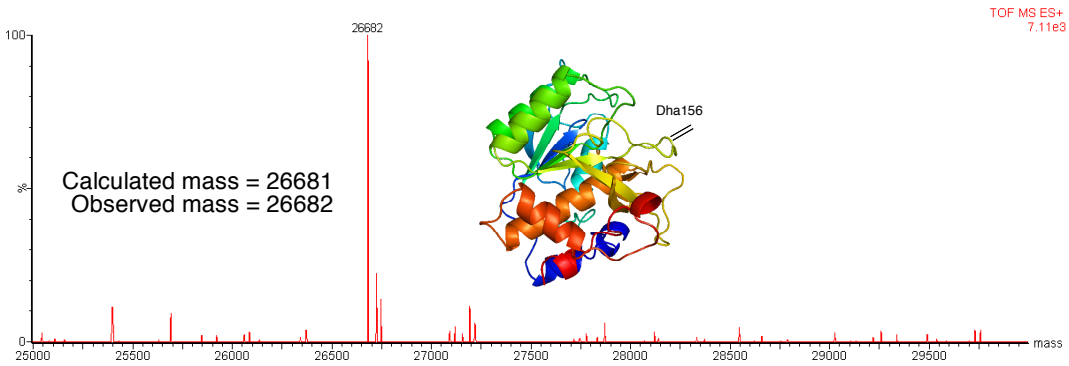
23

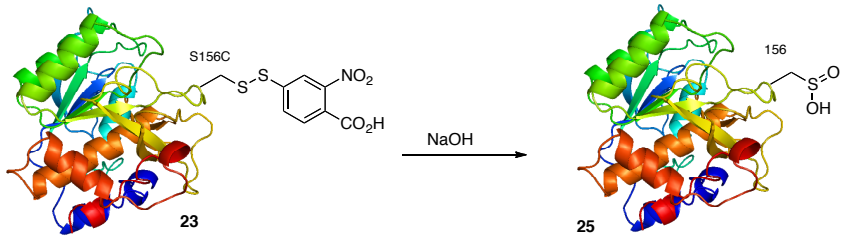
A 200 µL aliquot of SBL-S156C Ellman disulfide (**23**) (0.29 mg/mL in pH 8.0 sodium phosphate, 50 mM, 0.002 µmol) was added to a 1.0 mL plastic tube. Na_2CO_3 (5 mg) was added to the protein solution and the mixture vortexed to dissolve. A stock solution of HMPT was prepared by dissolving 2 µL in 250 µL of DMF. 25 µL of the HMPT solution was added to the protein solution and the reaction shaken at 4 ºC for 15 minutes. LC-MS analysis of the reaction mixture revealed full conversion to the HMPT phosphonium adduct **24** (calculated mass = 26877; observed mass = 26875). NaOH (50 µL of a 1.0M solution) was then added and then reaction shaken for 20 minutes at 4 ºC. LC-MS analysis revealed elimination to dehydroalanine (calculated mass = 26681, observed mass = 26682). ESI-MS are shown below. Above: HMPT adduct. Below: SBL-156Dha





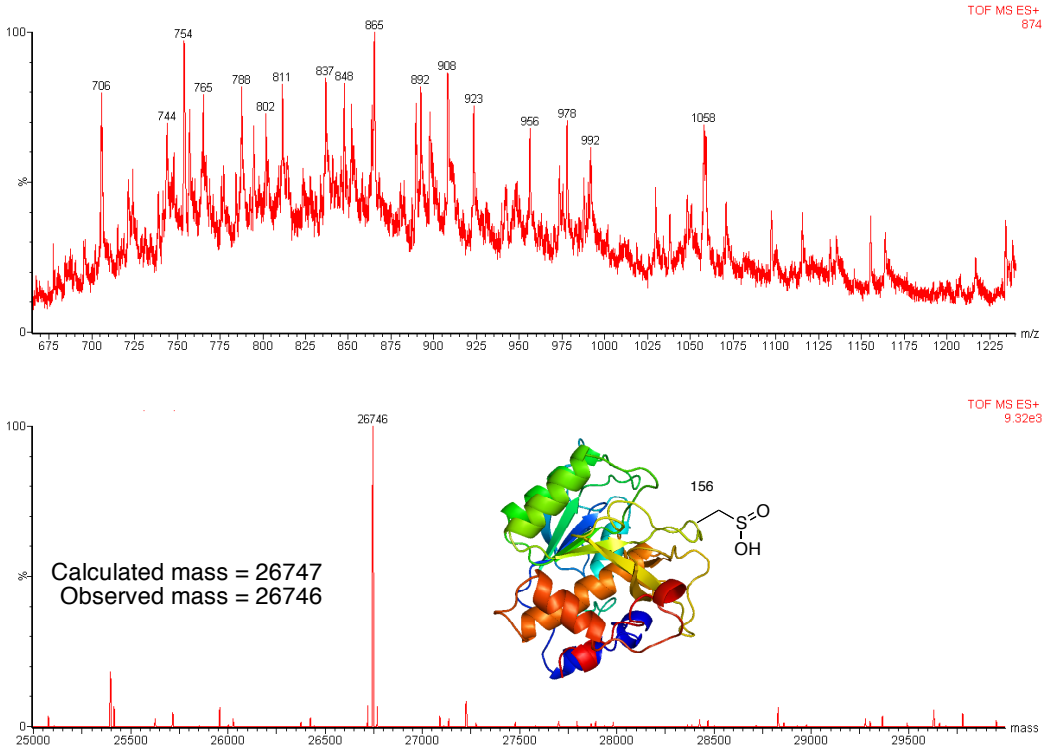




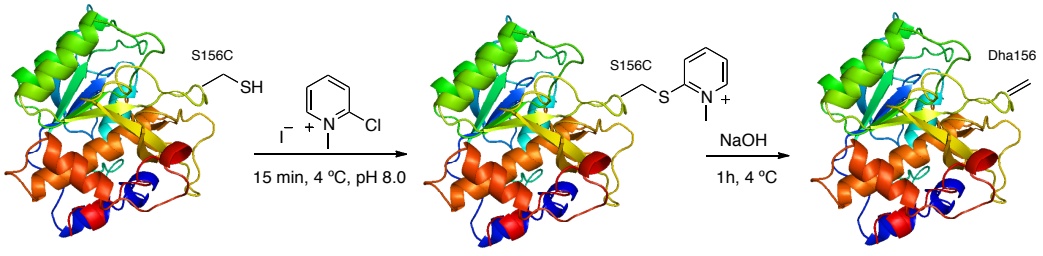


A 50 μL aliquot of SBL-S156C Ellman disulfide (0.29 mg/mL in pH 8.0 sodium phosphate, 50 mM, 0.5 nmol) was added to a 1.0 mL plastic tube. To this solution was added 50 μL of 1.0 M

NaOH. The mixture was shaken for 15 minutes at 4 ºC and then analyzed by LC-MS. No dehydroalanine was generated, only conversion to a protein with a mass corresponding to the sulfinic acid was observed (calculated mass = 26747, observed mass = 26746). ESI-MS are shown below.



Dha synthesis on SBL using Mukaiyama's Reagent

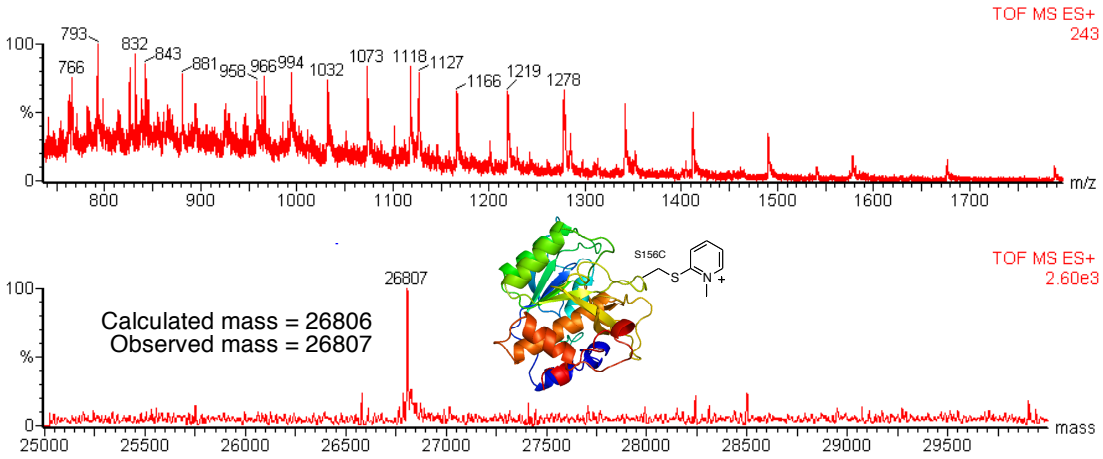


26

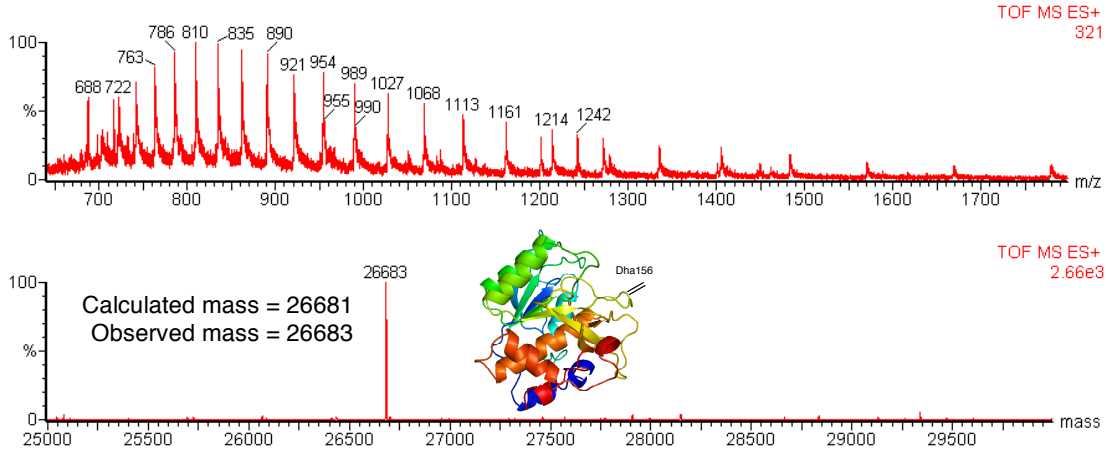
All manipulations were carried out in a cold room at 4 ºC. To a solution of SBL-S156C (500 µL of 0.7 mg/mL, 13.0 nmol) in sodium phosphate buffer (50 mM, pH 8.0), a freshly prepared solution of 2-chloro-1-methyl-pyridinium iodide (2 µL of 42 mg/mL, 328.0 nmol) in 50 mM sodium phosphate buffer (pH 8.0) was added and the resulting mixture vortexed for 30 seconds.

After 15 minutes of additional shaking, a 20 µL aliquot was analyzed directly by LC-MS and complete conversion to **26** was observed (calculated mass = 26806; observed mass = 26807). To the reaction mixture, 1M NaOH aq. (40 µL) was added and the resulting mixture vortexed for 30 seconds. After 1 h of shaking at 4 ºC, the reaction was analyzed directly by LC-MS and complete conversion to SBL-Dha was observed. The protein sample was passed through a PD minitrap to remove excess reagents (final concentration of 0.35 mg/mL), and the sample analyzed again by LC-MS (calculated mass = 26681; observed mass = 26683). ESI-MS are shown below.

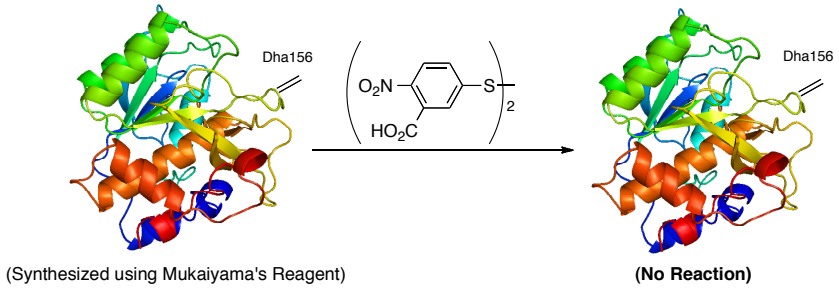
Mukaiyama Adduct (26):



Dehydroalanine:

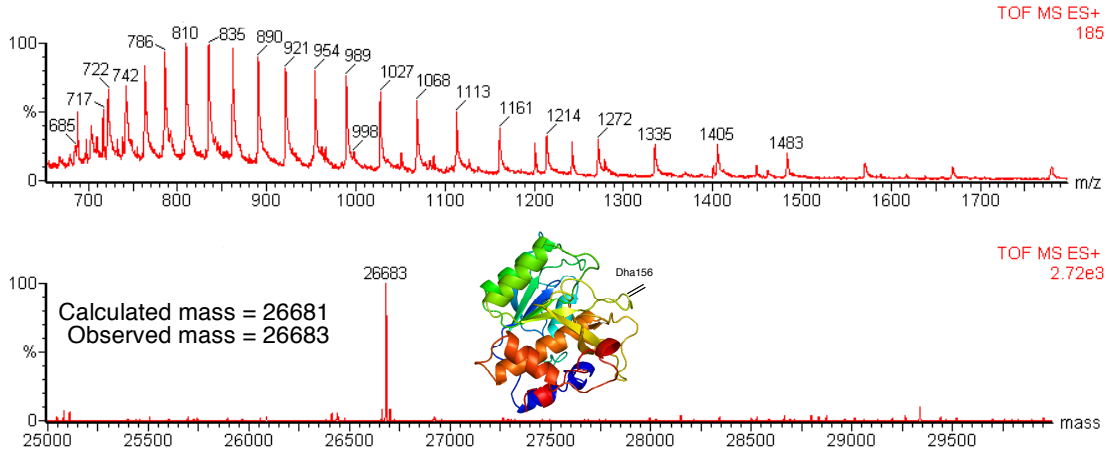


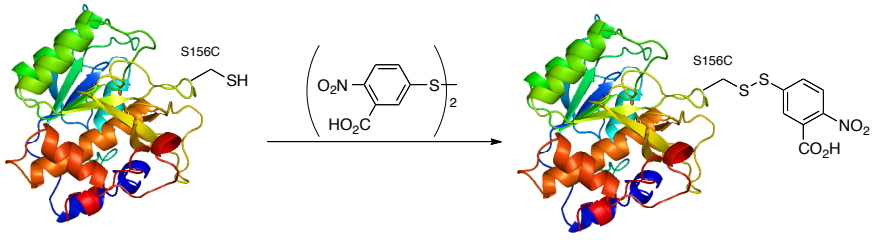
Ellman's Test to Confirm Reaction at Cysteine:



(Synthesized using Mukaiyama's Reagent) **(No Reaction)**

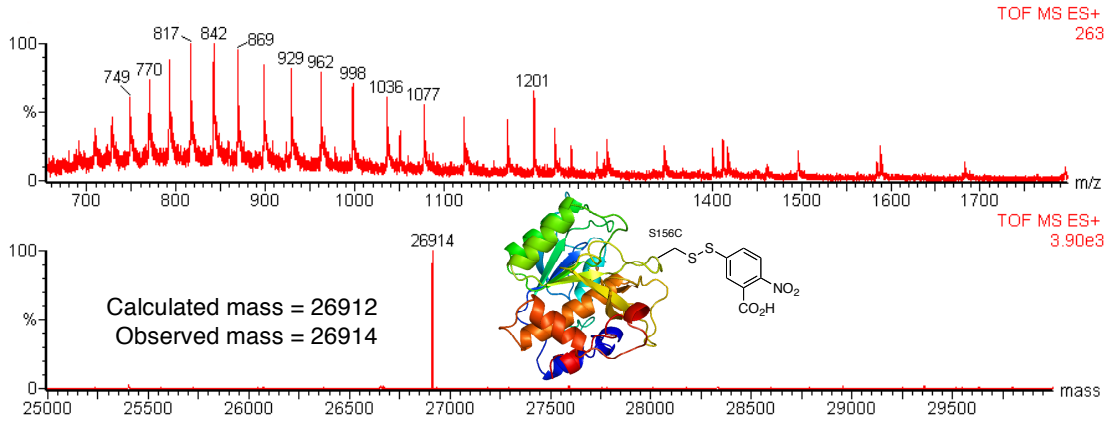
SBL-Dha prepared using Mukaiyama's reagent was prepared in sodium phosphate buffer (50 mM, pH 8.0) at a final concentration of 0.25 mg/mL. Ellman's solution was prepared by dissolving 0.74 mg in 100 µL of sodium phosphate buffer (50 mM, pH 8.0). A 10 µL aliquot of Ellman's solution (200 equivalents) was added to a 100 µL sample of SBL-Dha (0.25 mg/mL, sodium phosphate, pH 8.0, 50 mM). The reaction was vortexed, and shaken at room temperature for 15 minutes and then analyzed directly by LC-MS. No reaction was observed, indicating that all cysteine was consumed during the Dha synthesis (calculated mass = 26681; observed mass = 26683). Under identical conditions, SBL-S156C is converted to the corresponding disulfide (see next). ESI-MS are shown below.



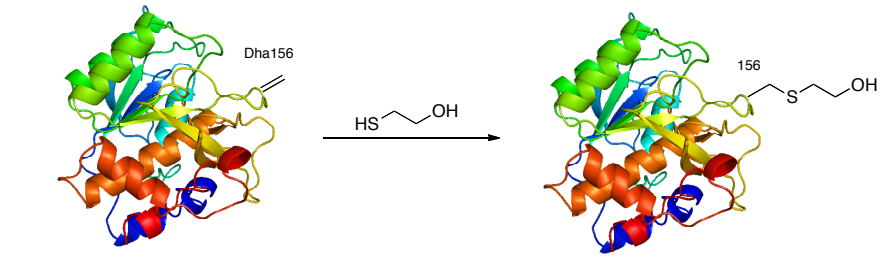


A 10 µL aliquot of the previously prepared Ellman's solution (200 equivalents) was added to a 100 µL sample of SBL-S156C (0.25 mg/mL, sodium phosphate, pH 8.0, 50 mM). The reaction

was vortexed, and shaked for at room temperature for 15 minutes and then analyzed directly by LCMS. In this case, full conversion to the Ellman disulfide is observed (calculated mass = 26912; observed mass = 26914). This reaction is the positive control for the Ellman's test above. ESI-MS are shown below.

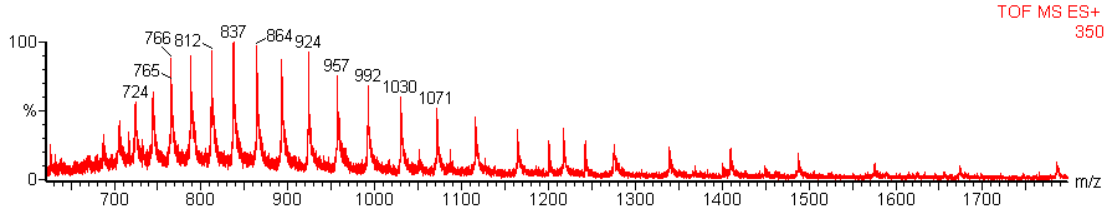


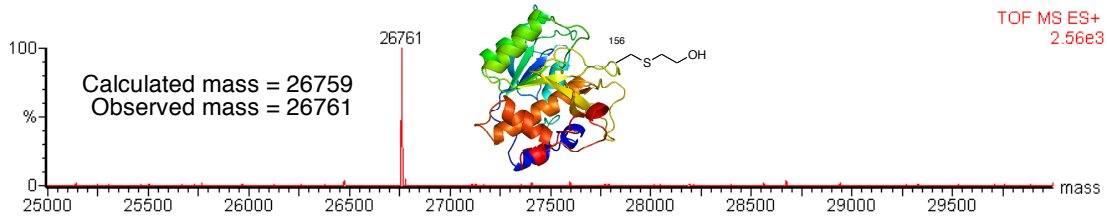
Chemical Test for Dehydroalanine: Addition of 2-Mercaptoethanol



(Synthesized using Mukaiyama's Reagent)

2-Mercaptoethanol (1 µL) was added to a 100 µL sample of SBL-Dha prepared using Mukaiyama's reagent (0.25 mg/mL, sodium phosphate, pH 8.0, 50 mM). The reaction was vortexed, and shaked for at room temperature for 30 minutes and then analyzed directly by LC-MS. Full conversion to the thioether was observed (calculated mass = 26759; observed mass = 26761). ESI-MS are shown below.

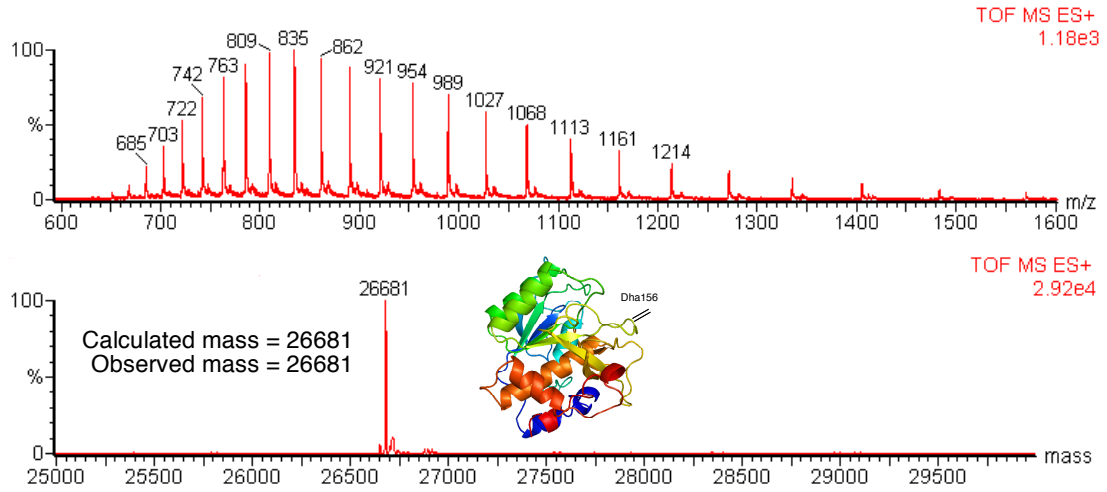




Synthesis of Dha on SBL-S156C using MSH

The following preparation of SBL-Dha156 using MSH and the resulting ESI-MS is representative of the published procedures.[29,30,31] The reader is referred to these publications for all related control experiements and other transformations of the dehydroalanine containing protein.

SBL-S156C was prepared as a 1.4 mg/mL solution in 50 mM sodium phosphate buffer (pH 8.0) and 1.00 mL (0.052 μmol) was added to a 1.50 mL plastic tube. MSH (1.2 mg, 5.6 μmol) was added as a solution in DMF (50 μL) and the reaction vortexed immediately upon addition. The homogenized sample was shaken at 4 ºC for 20 minutes before a 40 μL aliquot was analyzed by LC-MS, confirming full conversion of Cys156 to Dha156 (calculated mass = 26681; observed mass = 26681). This protein does not react with Ellman's reagent and a variety of thiols can be added by conjugate addition.[29,30,31] Representative ESI-MS are shown below.



Reaction of Np276 with MSH

The following data is representative of a non-selective reaction of MSH with a protein. For the expression and purification of Np276 and further use of this model protein, the reader is referred to the published procedures.[31] This data is taken from this previous report for interpretation in light of the results shown in Scheme 8 of this manuscript.

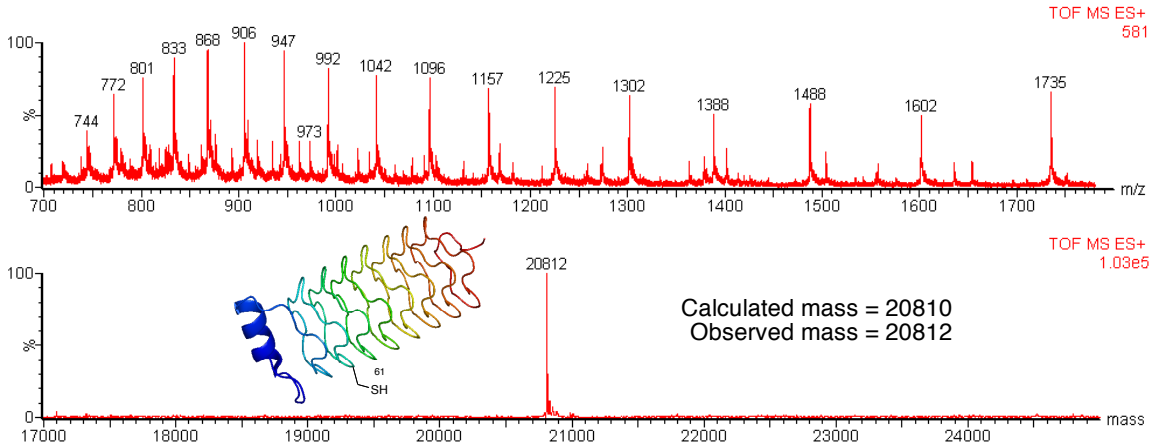
Np276 (PDB = 2J8K)

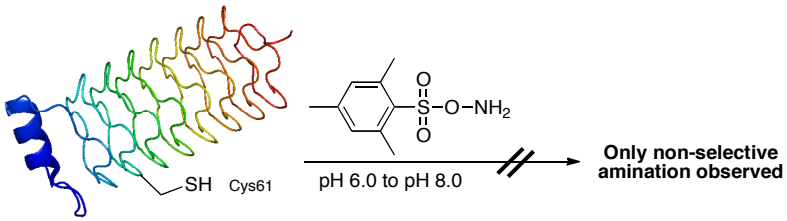
Sequence:

GSSHHHHHHSSGLVPRGSHIDVGKLRQLYAAGERDFSIVDLRGAVLENINLSGAILHGAC
LDEANLQQANLSRADLSGATLNGADLRGANLSKADLSDAILDNAILEGAILDEAVLNQA
NLKAANLEQAILSHANIREADLSEANLEAADLSGADLAIADLHQANLHQAALERANLTG
ANLEDANLEGTILEGGNNNLAT

Average Mass = 20810

ESI-MS for unmodified Np276 mutant is shown below

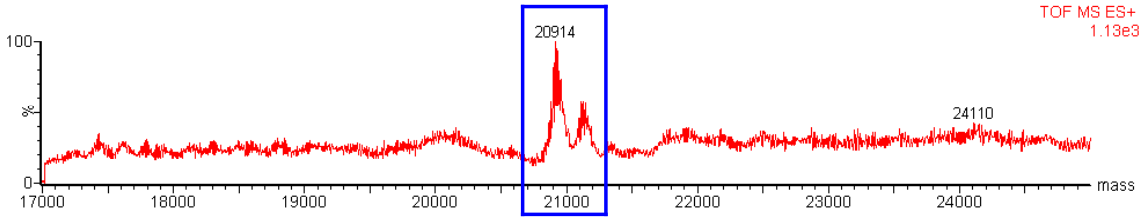




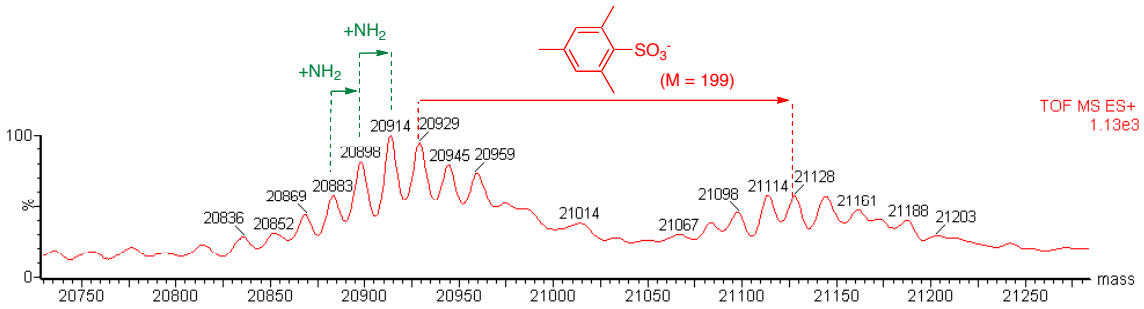
Np276-Cys61 (1.00 mL, c = 0.79 mg/mL, pH 8.0 sodium phosphate) was reduced with DTT (5 mg) and shaken at room temperature for 15 minutes. 500 µL of the reduced protein was passed through a PDmini column into pH 8.0 sodium phosphate buffer and the remaining 500 µL was passed through a PDmini column into pH 6.0 sodium phosphate buffer. The proteins solutions

were kept on ice. A stock solution of MSH was prepared by dissolving 3.4 mg in DMR (567 µL). Two reactions were set up: one at pH 8.0 and one at pH 6.0. Accordingly, 100 µL of the protein solution was mixed with 5 µL of the MSH solution at 4 ºC. The reaction was shaken at 4 ºC and then analyzed by LC-MS. A mixture of NH_2 adducts was observed at both pH values. Fewer NH_2 groups were observed at pH 6.0, but non-selective amination was still observed. ESI-MS are shown below.

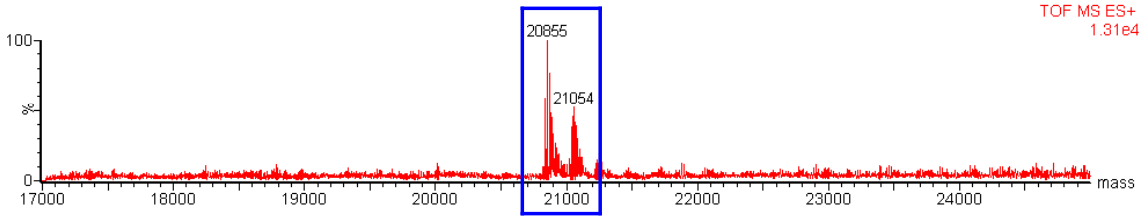
pH 8.0:



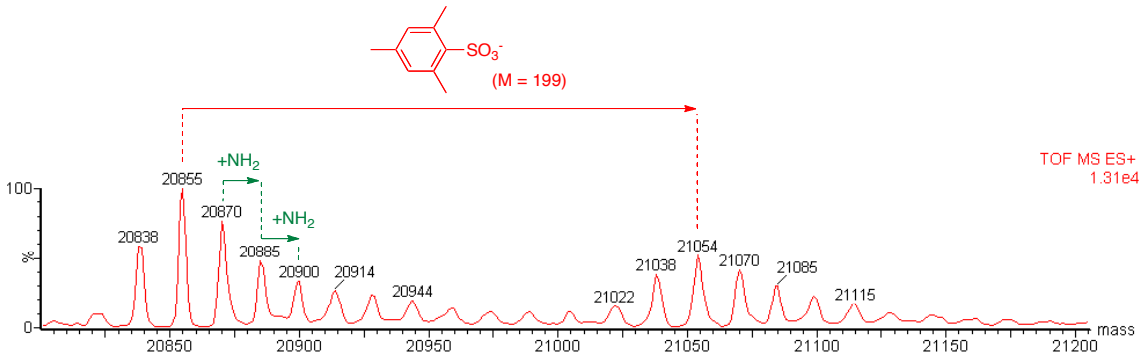
(Magnified region denoted in blue above): Representative peaks separated by units of "NH_2" are denoted in green. Two clusters of peaks are also observed that are separated by the mass of the mesitylene sulfonate anion (red).



pH 6.0:

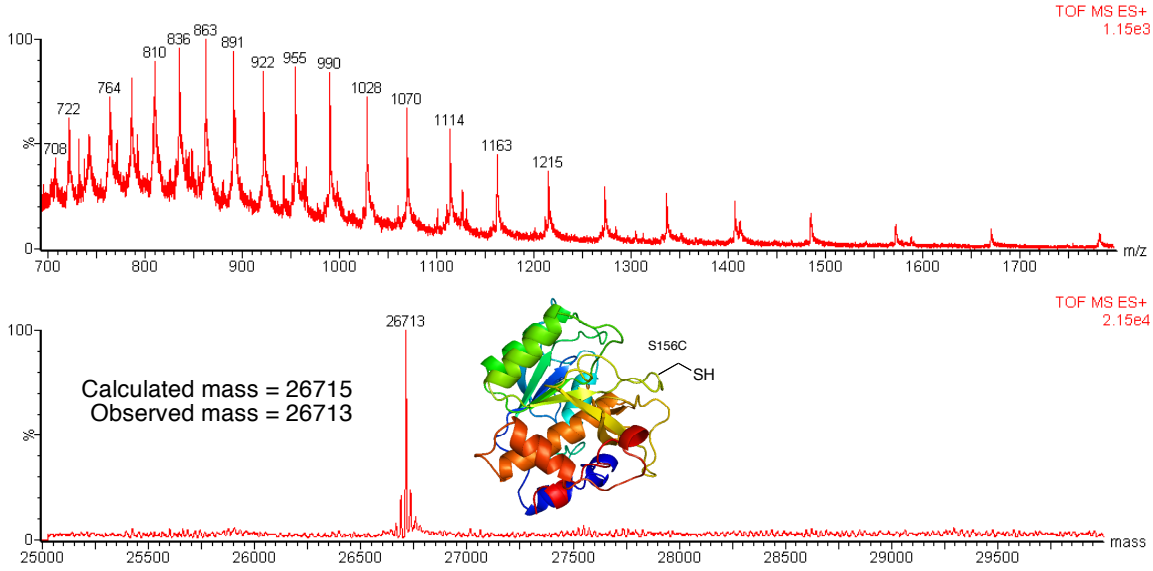


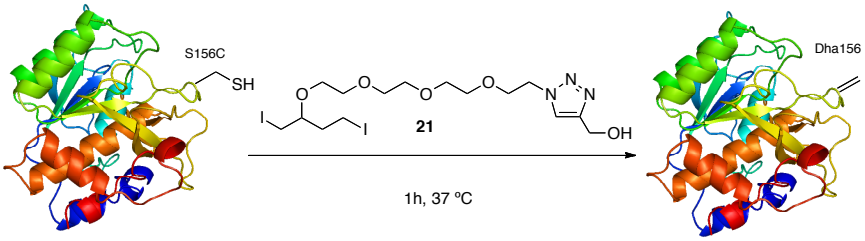
(Magnified region denoted in blue above): Representative peaks separated by units of "NH_2" are denoted in green. Two clusters of peaks are also observed that are separated by the mass of the mesitylene sulfonate anion (red).



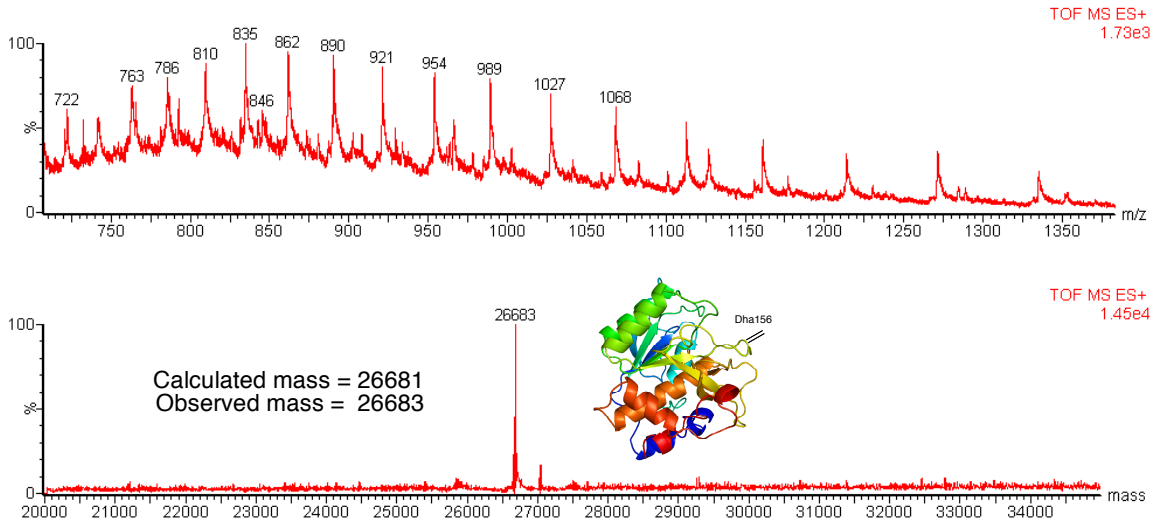
Protein Reactions using Dialkylation-Elimination of Cysteine to Dehydroalanine

SBL (S156C) was prepared at 2.0 mg/mL in pH 8.0 sodium phosphate buffer (50 mM) and reduced by the addition of DTT (6 mg). After shaking at room temperature for 20 minutes, the solution was passed through a PD minitrap to remove excess DTT. The protein was eluted with sodium phosphate buffer (pH 8.0, 50 mM). The final concentration was 1.0 mg/mL. The protein was stored on ice until needed. ESI-MS are shown below.

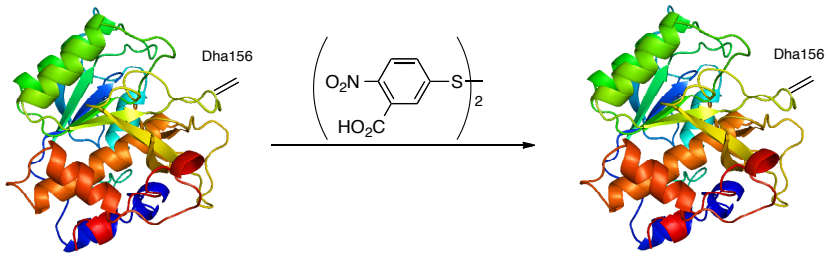




A solution of the di-iodide triazole **21** was prepared by dissolving 10.9 mg (0.019 mmol) in 52.6 µL of DMF. A 500 µL solution of SBL-S156C (1 mg / mL in pH 8.0 sodium phosphate buffer, 19 nmol protein) was added to the triazole solution and the mixture was vortexed immediately. The reaction was shaken at 37 ºC for 1 hour. After this time, any precipitate was removed by centrifugation (2 min, 14K g). The supernatant was analyzed by LC-MS, revealing complete conversion to dehydroalanine (calculated mass = 26681; observed mass = 26683 found). LC-MS is show below.



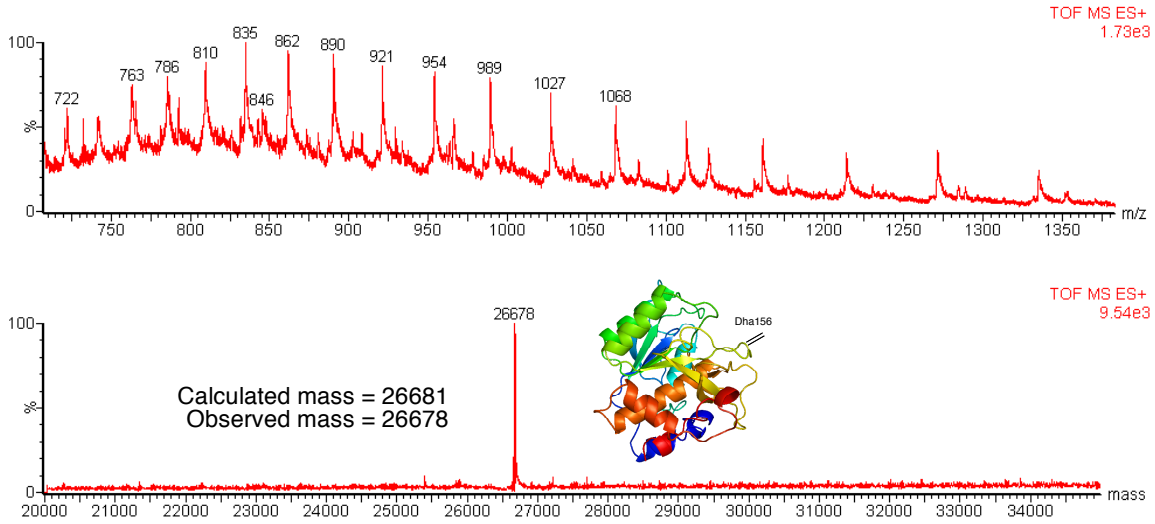
Ellman's Test to Confirm Reaction at Cysteine:

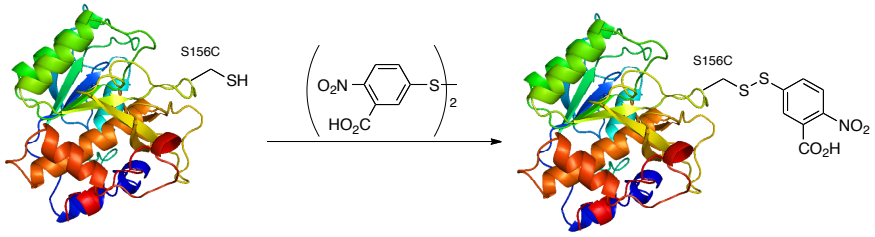


(Synthesized using **21**) **(No Reaction)**

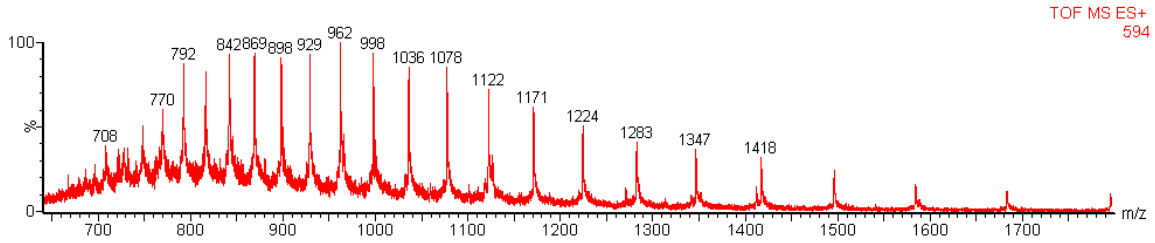
Ellman's solution was prepared by dissolving 0.4 mg in 108 µL of sodium phosphate buffer (pH 8.0, 50 mM). A 10 µL aliquot of Ellman's solution was added to a 50 µL sample of SBL-Dha

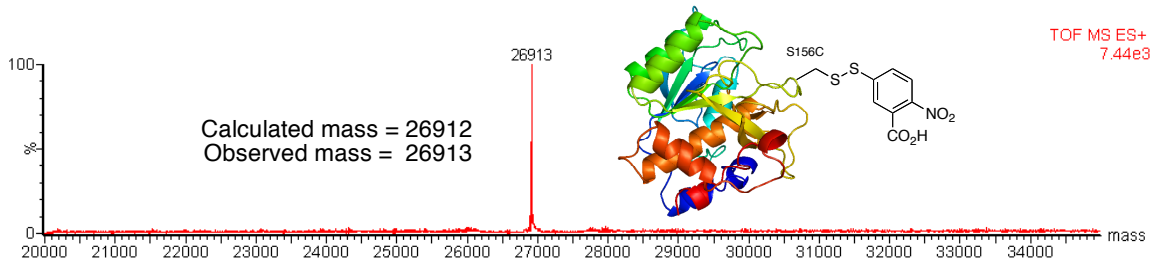
prepared above using **21** (0.5 mg/mL, pH 8.0 sodium phosphate). The reaction was shaken at room temperature for 15 minutes and then analyzed by LC-MS. No reaction was observed, indicating all cysteine was consumed in the elimination. (calculated mass = 26681; observed mass = 26678). Under identical conditions, SBL-S156C is converted to the Ellman disulfide (see next). ESI-MS are shown below.



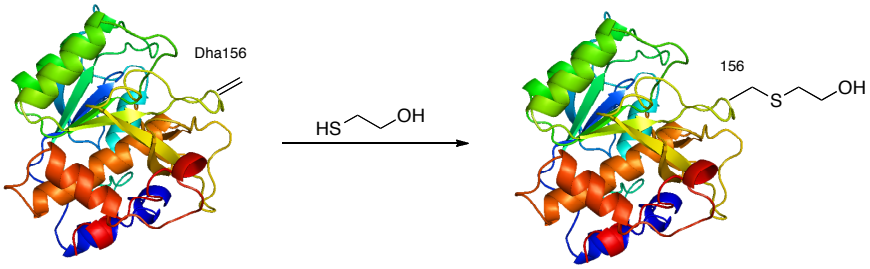


A solution of SBL (S156C) was prepared at 0.5 mg/mL in pH 8.0 sodium phosphate buffer (50 mM). A 10 µL aliquot of the Ellman's solution prepared in the previous reaction was added to 50 µL of SBL-(S156C). The reaction was shaken at room temperature for 15 minutes and then analyzed by LC-MS. Full conversion to the disulfide was observed (calculated mass = 26912; observed mass = 26913). ESI-MS are shown below.



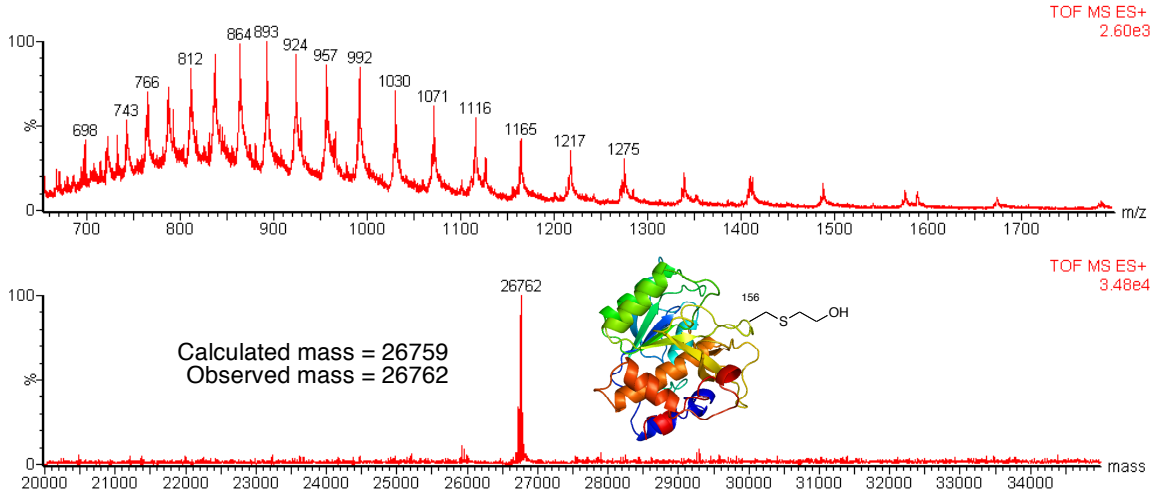


Chemical Test for Dehydroalanine: Addition of 2-Mercaptoethanol

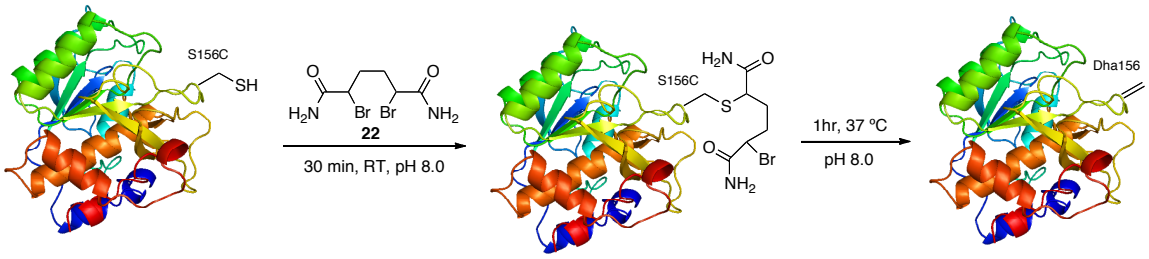


(Synthesized using **21**)

SBL-156Dha was prepared using di-iodide triazole **21** as described above. A 50 μL aliquot of the SBL-156Dha was mixed with 3.3 μL of 2-mercaptoethanol. The reaction was incubated at room temperature for 30 minutes and then analyzed by LC-MS. Full conversion to the thioether was observed (calculated mass = 26759; observed mass = 26762). ESI-MS are shown below.

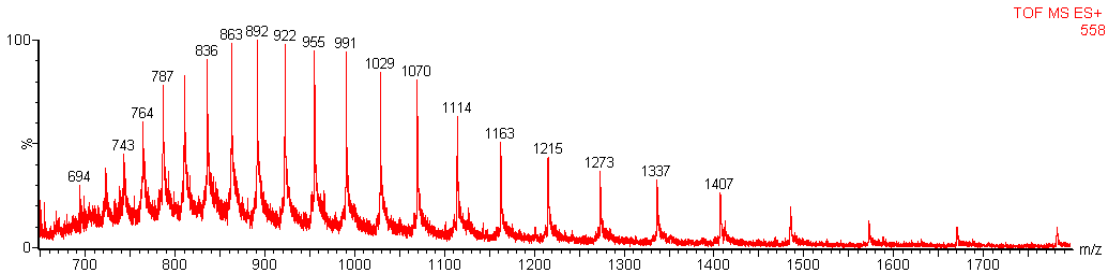


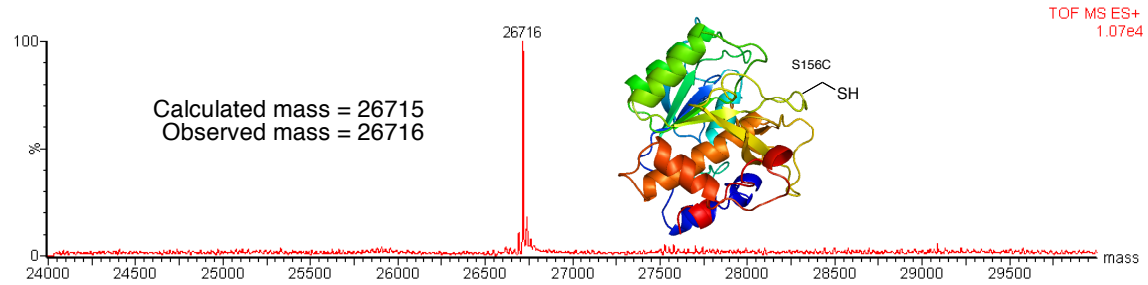
SBL-156Dha synthesis using 22



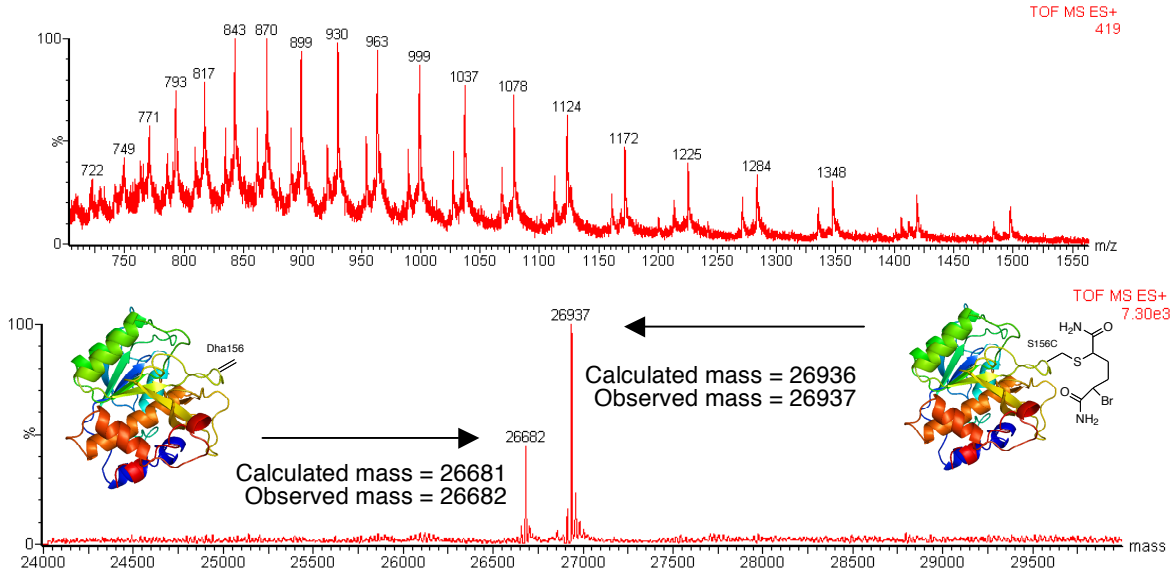
SBL-S156C was prepared at 1.0 mg/mL in sodium phosphate buffer (pH 8.0, 50 mM). DTT (1.4 mg) was added as a solid to a 500 µL aliquot of the protein solution to reduce any contaminant disulfide. After 15 minutes of incubation at room temperature, the DTT was removed by passing the protein solution through a PD minitrap (GE Healthcare), eluting with 1 mL of sodium phosphate buffer (pH 8.0, 50 mM). A 100 µL aliquot of the reduced protein (0.5 mg/mL, 0.002 µmol) was used for the elimation reaction. Accordingly, a stock solution of di-bromide **22** was prepared by dissolving 35.5 mg in 418 µL DMF. A 10 µL aliquot of the di-bromide solution (3 µmol) was added to the 100 µL protein solution at room temperature. The reaction was vortexed to homogenize and shaken at room temperature for 30 minutes. After this time, solids were removed by centrifugation (1 min, 14K g). (Note: some di-bromide crystallizes / precipitates from the reaction mixture). The supernatant was analyzed by LC-MS, revealing two products: alkylated, uncyclized intermediate (calculated mass = 26936; observed mass = 26937) and dehydroalanine (calculated mass = 26681; observed mass = 26682). The reaction was then shaken at 37 ºC for an hour to complete the cyclization and elimination. After this time, solids were removed by centrifugation and the supernatant analyzed by LC-MS. A single protein was detected with a mass corresponding to the dehydroalanine containing protein (calculated mass = 26681; observed mass = 26682). ESI-MS for the entire reaction sequence are shown below.

t = 0 (Starting material SBL-S156C)

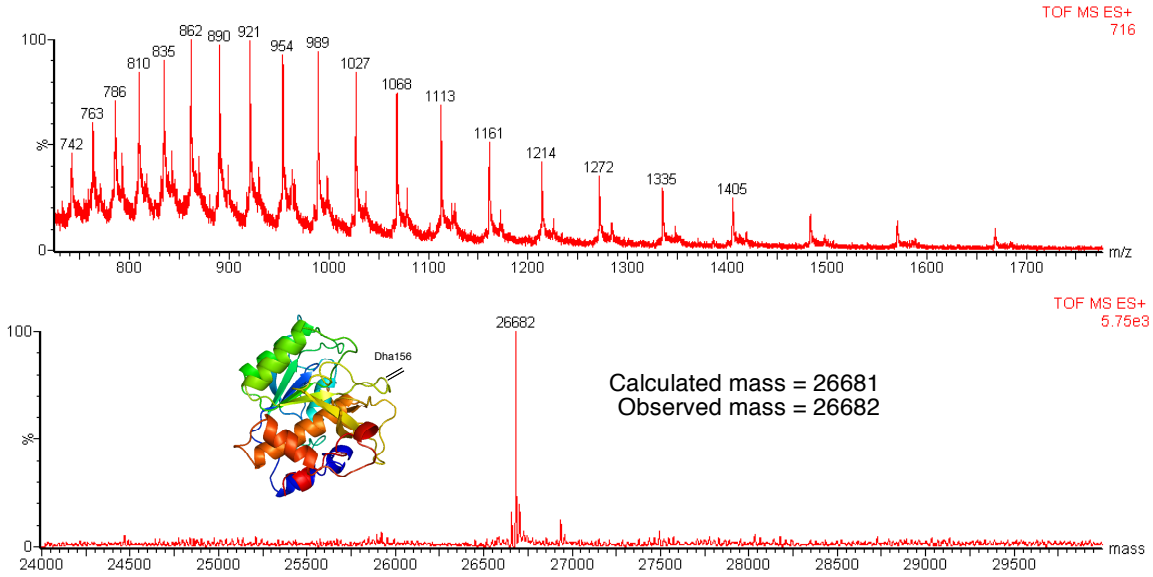




t = 30 min, RT

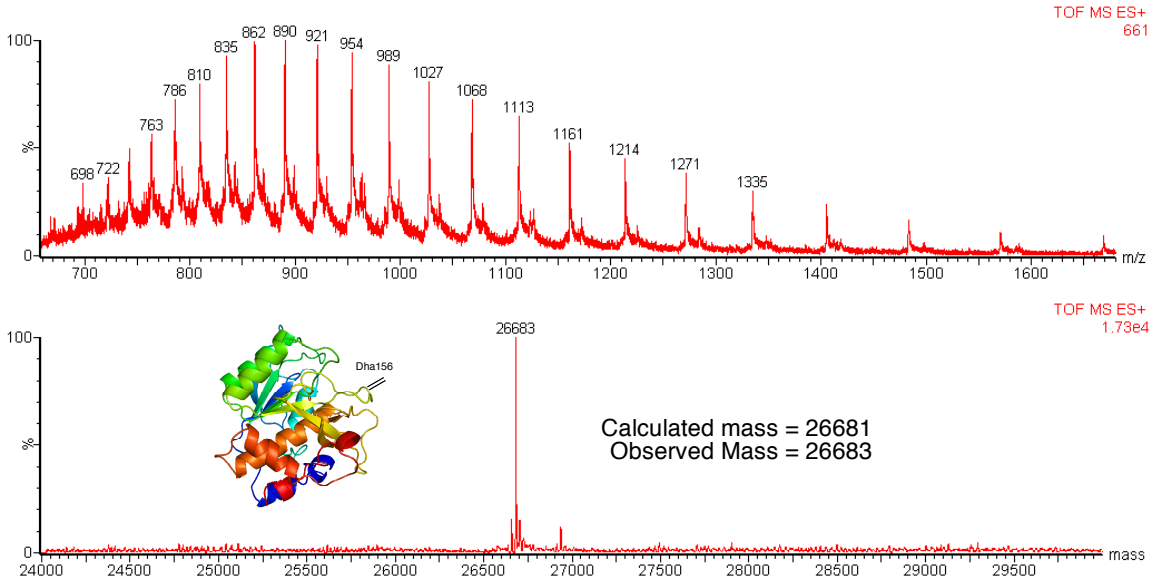


t = 90 min (after 60 min at 37 °C)

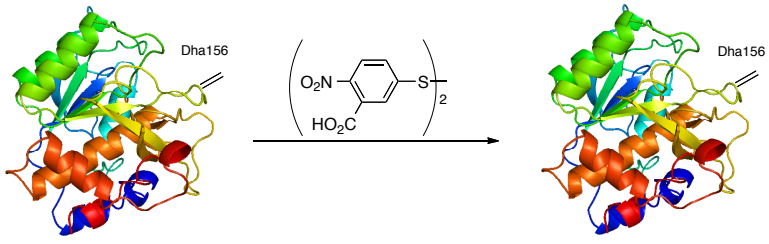


The procedure was repeated to obtain more SBL-Dha:

A 450 μL aliquot of SBL-S156C (c = 0.5 mg/mL, pH 8.0 sodium phosphate buffer) was warmed to room temperature. A 45 μL aliquot of the di-bromide solution prepared above was added to the protein. The reaction was shaken at room temperature for 30 minutes and then at 37 ºC for 1 hour. After this time, solids were removed by centrifugation (1 min, 14K g) and the solid was passed through a PD minitrap, eluting with 1 mL of the reaction buffer (pH 8.0 sodium phosphate, 50 mM). LC-MS analysis of the product revealed a mass corresponding to the dehydroalanine containing protein (calculated mass = 26681; observed mass = 26682). ESI-MS are shown below.



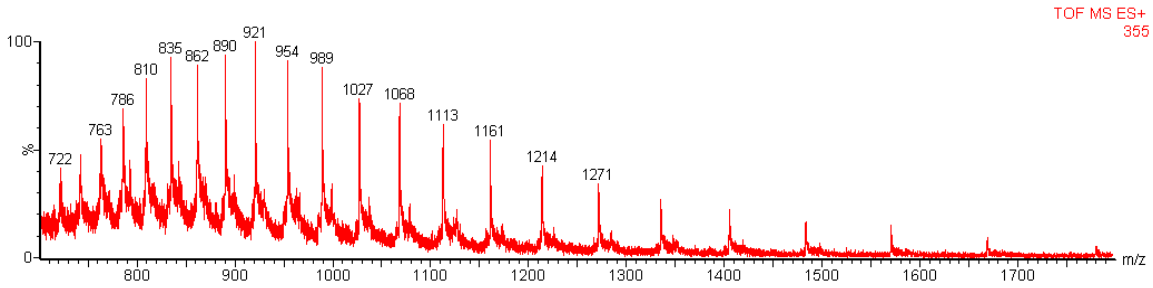
Ellman's Test to Confirm Reaction at Cysteine:

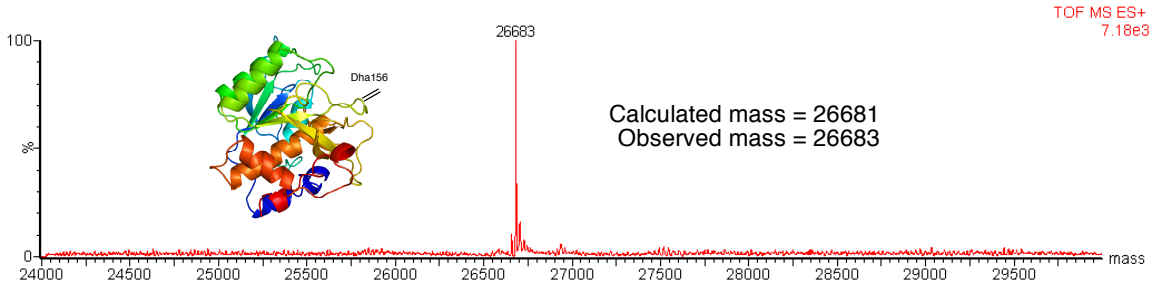


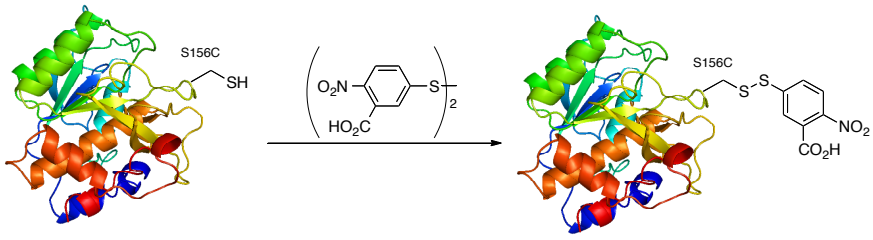
(Synthesized using **22**) **(No Reaction)**

Ellman's assay was carried out to verify consumption of free thiol, and hence reaction at cysteine. Accordingly, a stock solution of Ellman's reagent was prepared by dissolving 0.5 mg in 10.2 mL of sodium phosphate buffer (pH 8.0, 50 mM). 2 μL of the Ellman's solution was added to a 30 μL aliquot of the SBL-Dha solution (0.22 mg/mL) prepared above. The solution was shaken for 15

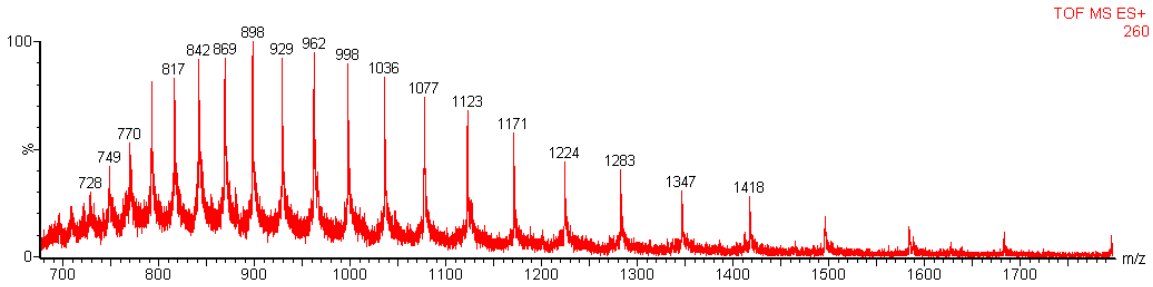
minutes at room temperature and then analyzed by LC-MS. No reaction was observed (calculated mass = 26681; observed mass = 26683). Under the same reaction conditions, SBL-S156C is converted to the Ellman disulfide (see next). ESI-MS are shown below.

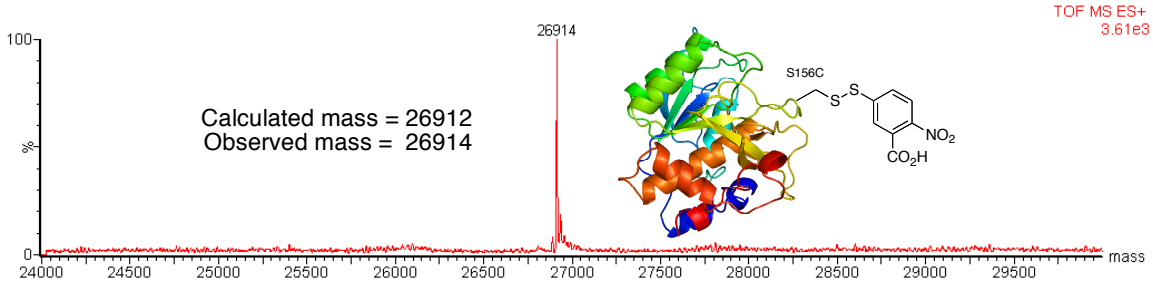




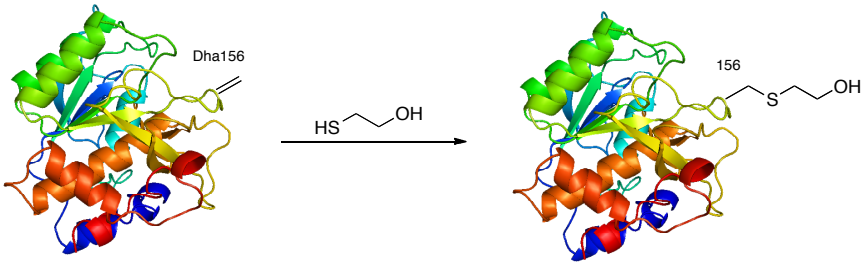


SBL-S156C was prepared at 0.22 mg/mL in pH 8.0 sodium phosphate buffer. A 2 μL aliquot of the Ellman's reagent solution prepared above was added to 30 μL of SBL-Dha solution. The reaction was shaken 15 minutes at room temperature, after which time full conversion to the corresponding disulfide was observed (calculated mass = 26912; observed mass = 26914). ESI-MS are shown below.



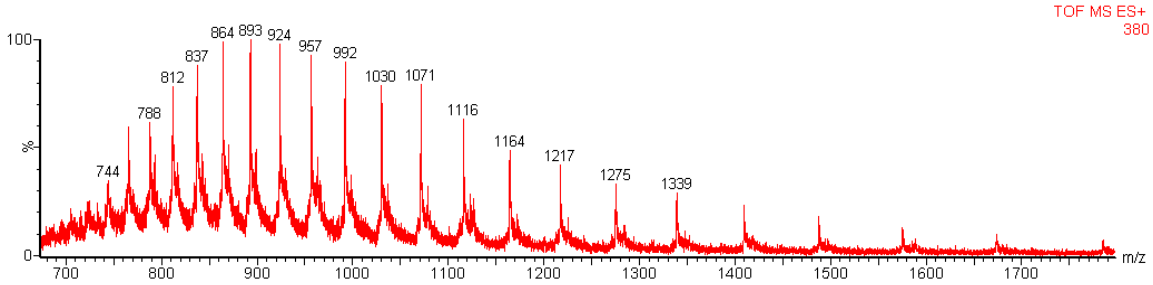


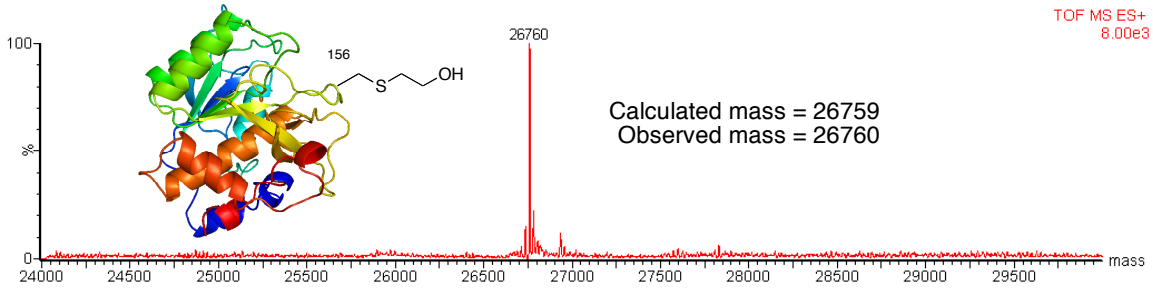
Chemical Test for Dehydroalanine: Addition of 2-Mercaptoethanol



(Synthesized using **22**)

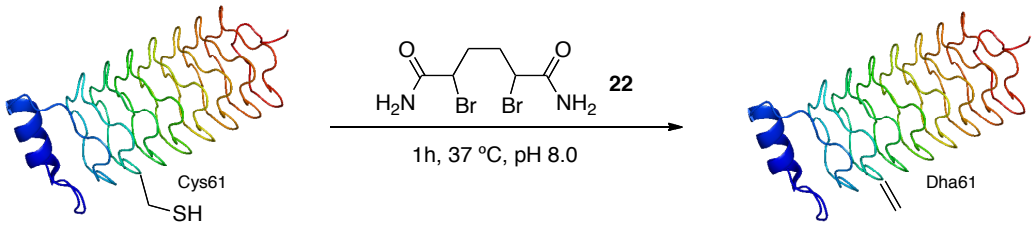
The presence of dehydroalanine was corroborated by the addition of β-mercaptoethanol. Accordingly, a 25 μL aliquot of the SBL-Dha protein (0.22 mg/mL) was mixed with 0.7 μL of β-mercaptoethanol. The reaction was shaken at room temperature for 30 minutes and then analyzed by LC-MS. Full conversion to the corresponding thioether was observed (calculated mass = 26759; observed mass = 26760).



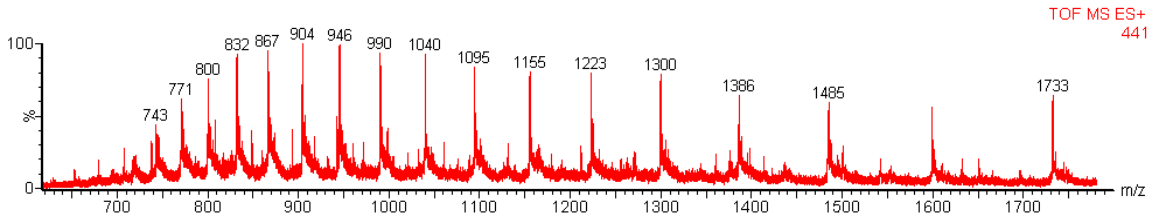


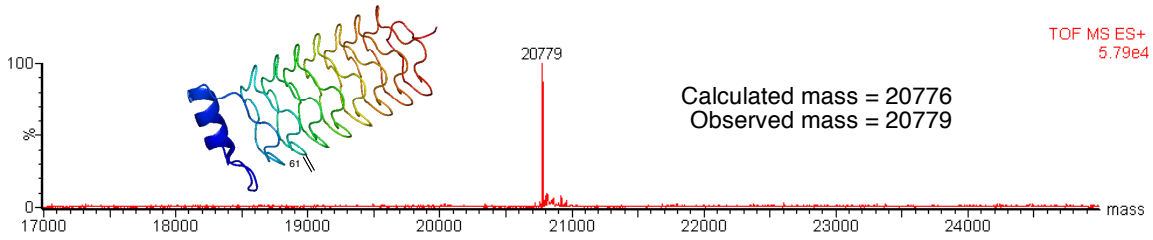
Dha formation on Np276 using di-bromide 22

This procedure is representative for that previously published for using reagent **22** on Np276.[31] This data is included here for comparison to the unselective reaction of Np276 with MSH and for a protocol for the conversion of hindered cysteine to dehydroalanine. For the expression and purification of Np276, the reader is reffered to the previously published procedure.[31]

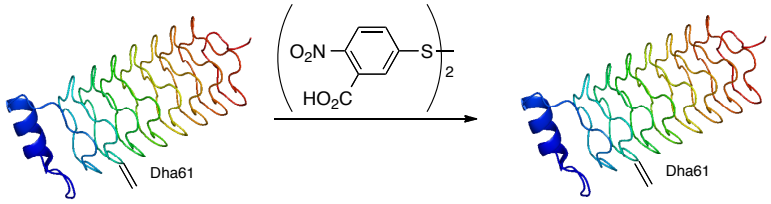


Np276-Cys61 (2.5 mL, 1.1 mg / mL in pH 8.0 sodium phosphate, 50 mM) was reduced by the addition of 10 mg DTT. The solution was shaken at room temperature for 15 minutes to reduce any contaminant disulfide. After this time, the protein solution was passed through a PD10 column (GE Healthcare), eluting with the same buffer. The resulting protein solution was used immediately. Accordingly, 2.5 mL of the Np276 protein (c = 0.79 mg/mL) was added to a 15 mL plastic tube and kept at room temperature. A stock solution of di-bromide **22** was prepared by dissolving 218 mg in 1.91 mL DMF. A 125 µL aliquot of the di-bromide solution was added to the protein solution and the reaction was vortexed to homogenize and immediately placed in a 37 ºC incubator with shaking. After 15 minutes, an additional 125 µL aliquot of the di-bromide solution was added to the reaction. After another 15 minutes, a third and final 125 µL aliquot of the di-bromide solution was added. The reaction was shaken at 37 ºC for a final 30 minutes and then analyzed by LC-MS. A protein mass corresponding to the Dha protein was observed (calculated mass = 20776; observed mass = 20779). Precipitated di-bromide was removed by centrifugation and the protein solution was stored on ice. ESI-MS are shown below.





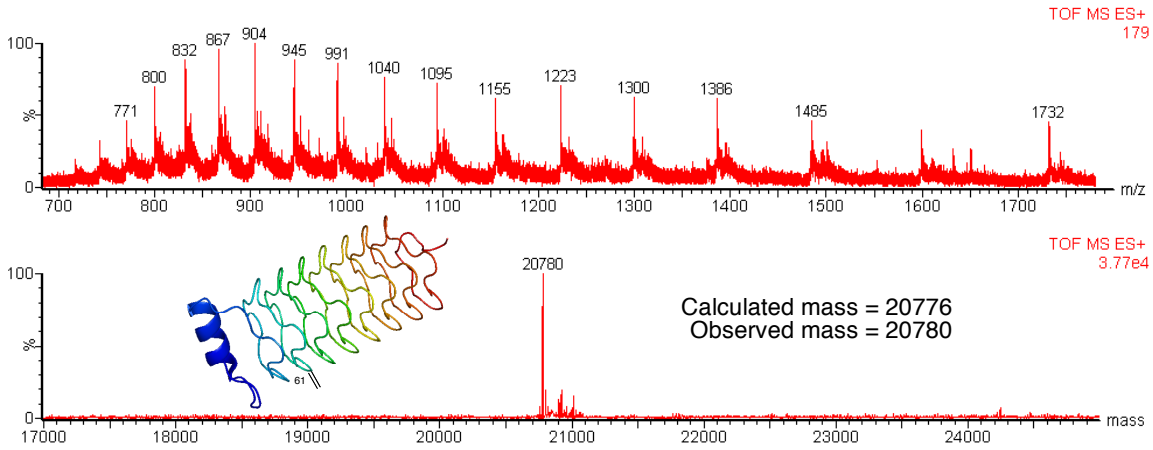
Ellman's Test to Confirm Reaction at Cysteine:

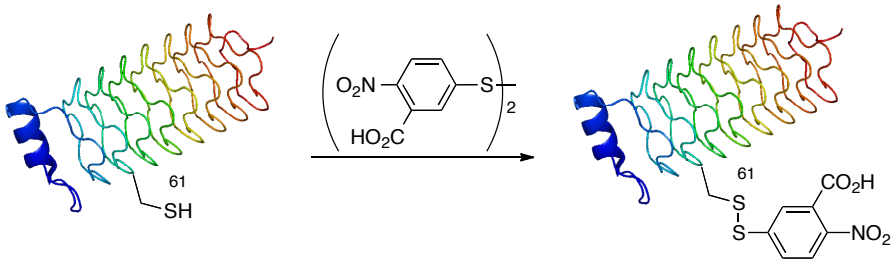


(Synthesized using **22**)

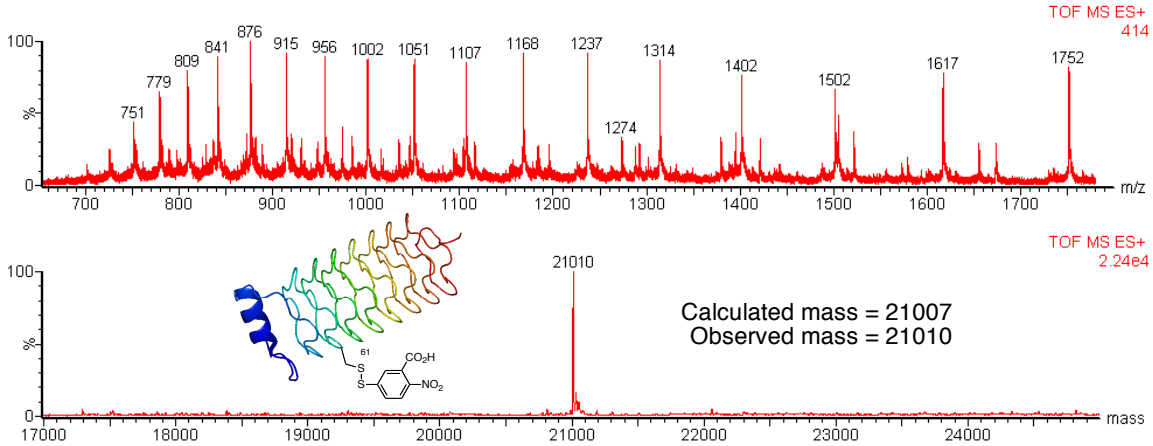
(No Reaction)

A stock solution of Ellman's reagent was prepared by dissolving 0.5 mg in 250 µL of sodium phosphate buffer (pH 8.0, 50 mM). A 140 µL aliquot of the dehydroalanine containing protein (0.79 mg/mL in pH 8.0 sodium phosphate buffer) was mixed with 10 µL of the Ellman's solution. The solution was incubated at room temperature for 15 minutes and then analyzed by LC-MS. No reaction was observed, indicating complete consumption of cysteine during the elimination reaction (calculated mass = 20776 calculated; observed mass = 20780). ESI-MS are shown below. Under identical conditions, Np276-Cys61 is converted to the Ellman disulfide (see next).

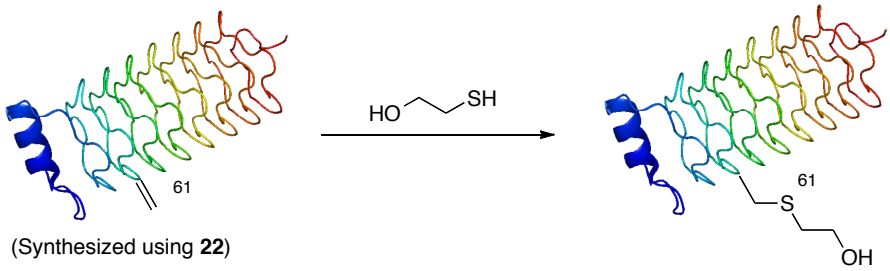




Np276-Cys61 (140 µL of a 0.79 mg/mL solution in pH 8.0 sodium phosphate buffer, 50 mM) was mixed with 10 µL of the Ellman's reagent solution prepared above. The reaction was shaken at room temperature for 15 minutes and then analyzed by LC-MS. Full conversion to the Ellman disulfide was observed (calculated mass = 21007; observed mass = 21010). ESI-MS are shown below.

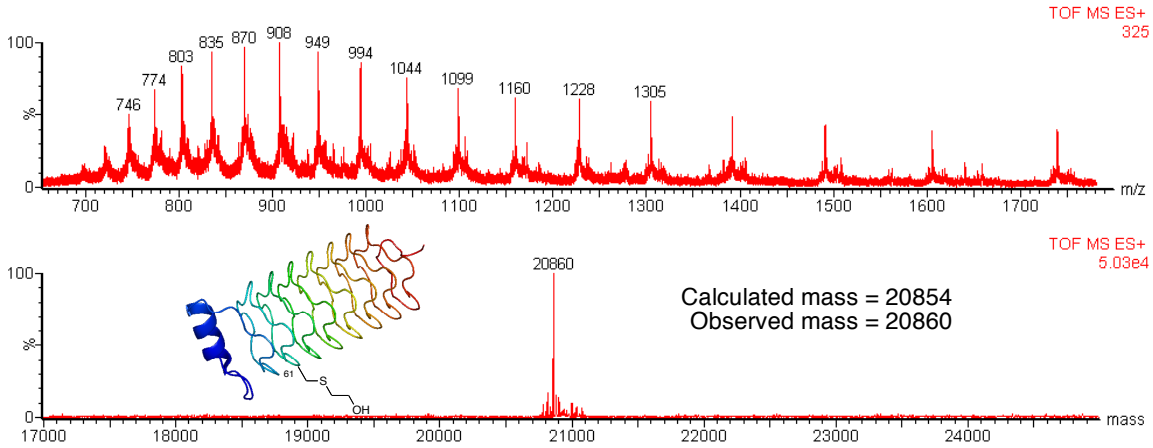


Chemical Test for Dehydroalanine: Addition of 2-Mercaptoethanol

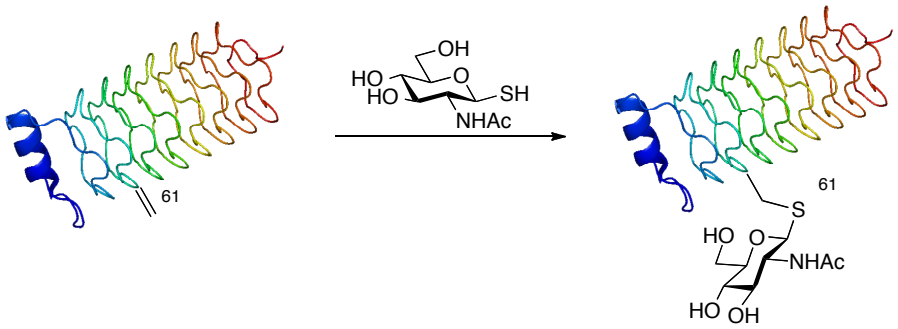


(Synthesized using **22**)

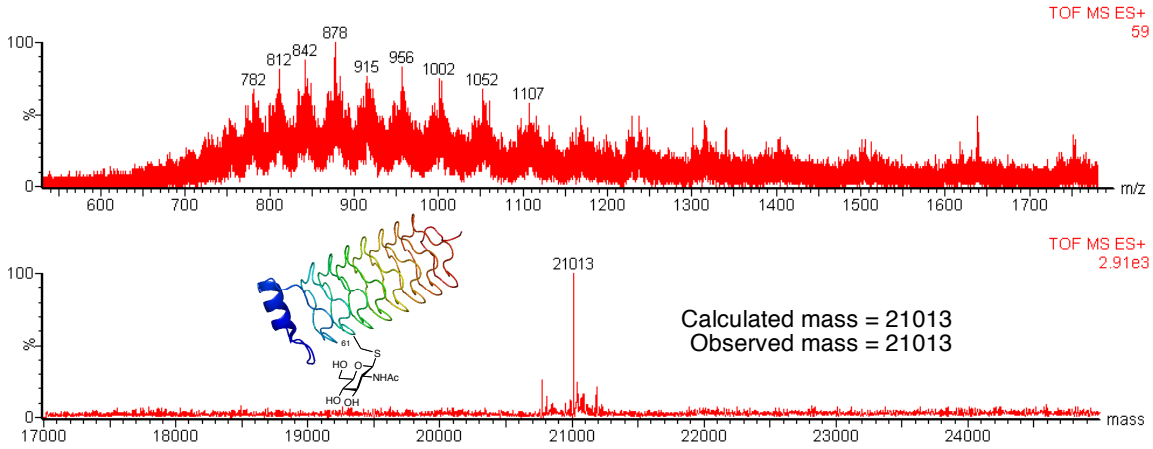
A 100 µL aliquot of the Dha containing Np276 was mixed with 5 µL of 2-mercaptoethanol. The reaction was shaken at 37 ºC for 1 hour. After this time, full conversion to the thioether was observed by LC-MS (calculated mass = 20854; observed mass = 20860). ESI-MS are shown below.



Addition of GlcNAc thiol 27 to Dha on Np276



A 1.00 mL aliquot of the Dha containing Np276 (c = 0.79 mg/mL) was mixed with 135 mg of GlcNAc thiol **27**. The reaction was shaken at 37 ºC for 2 hours. After this time, small molecules were removed by dialysis (2 × 4L pH 7.0 sodium phosphate, 50 mM). Full conversion to the thioether was observed by LCMS (calculated mass = 21013; observed mass = 21013). ESI-MS are shown below.

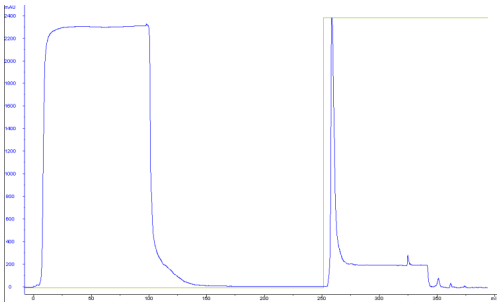


Expression and Purification of single-domain antibody cAb-Lys3

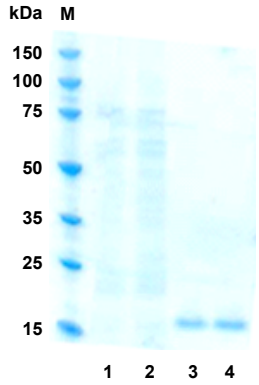
The same procedure was used for cAb-Lys3 (wild type) and cAb-Lys3-A104C:

CHO cells were grown in 1 L CD CHO medium in a 3 L flask for 1 week at 37 °C with shaking. Cells were harvested by centrifugation (450 g, 15 minutes, 4 °C) and the supernatant decanted. Pelleted cells were resuspended in Earle's balanced salt solution (EBSS 20 mL, Sigma Aldrich), and then harvested by centrifugation (450 g, 15 minutes, 4 °C). Pelleted cells were then resuspended in EBSS (2 mL) containing the vector for cAb-Lys3 (4 mg total DNA; the vector is a proprietary construct supplied by UCB). The solution was made up to 10 mL with EBSS and kept on ice. This was split into 800 μL aliquots and the cells electroporated at 300 V. Transfected cells were added to Gibco CD CHO medium (Invitrogen), and 1 L medium was incubated with shaking in a 3 L flask at 37 °C for 1 day, and then at 32 °C for 13 days. CD CHO medium was prepared by preheating all components to 37 °C, followed by the addition 100× GlutaMAX (10 mL, Invitrogen) and antibiotic-antimycotic (2 mL, Invitrogen). Cells were harvested by centrifugation (450 g, 15 minutes, 4 °C), and the supernatant taken to the purification step. Protein purification was carried out on an Äkta FPLC coupled to a Frac-900 fraction collector. A 5 mL HisTrap HP (GE Healthcare) was equilibrated with 5 column volumes of binding buffer (50 mM sodium phosphate, 300 mM NaCl, 10 mM imidazole, pH 8.0) before sample loading. The column was washed with 2 column volumes of binding buffer to remove excess unbound protein before a step to 100 % elution buffer (50 mM sodium phosphate, 300 mM NaCl, 250 mM imidazole, pH 8.0) over 5 column volumes. Protein was detected by UV absorbance at 280 nm. A flow rate of 5 mL/min was used. Flow through and eluted fractions were analysed by SDS-PAGE (4–12 % NuPAGE pre-cast Bis-Tris gel (Invitrogen), MES running buffer (Invitrogen)). Fractions containing protein were concentrated (Vivaspin, Sartorius Stedim) and buffer exchanged (PD minitrap, GE Healthcare) into PBS, pH 8.0. 90 mg each of cAb-Lys3 (wild type) and cAb-Lys3-A104C was obtained in total. Protein was split into 1 mg/mL aliquots, snap frozen in liquid N_2 and stored at – 80 °C. FPLC data and SDS-PAGE are shown below for cAb-Lys3 (wild type) and cAb-Lys3-A104C. ESI-MS and sequence data are also shown below.

cAb-Lys3 (wild type)

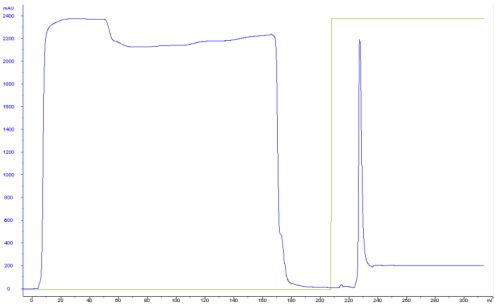


FPLC: UV at 280 nm (blue), Imidazole gradient (green)

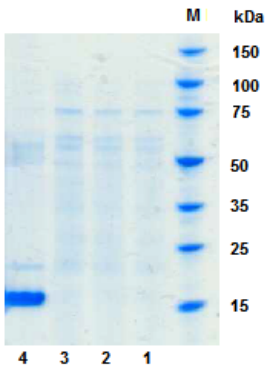


SDS-PAGE: Flow through (1,2); Elution (3,4)

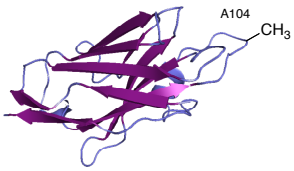
cAb-Lys3-A104C



FPLC: UV at 280 nm (blue), Imidazole gradient (green)



SDS-PAGE: Flow through (1-3); Elution (4)



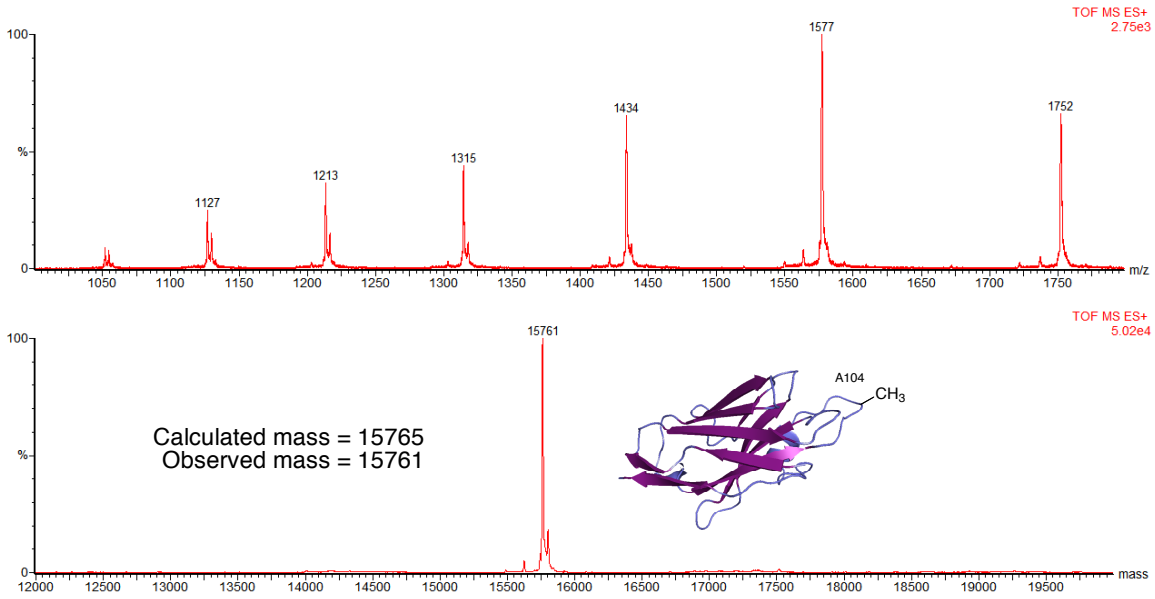
cAb-Lys3 (wild type) (PDB = 1MEL)

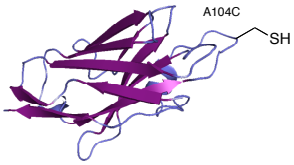
Sequence:

DVQLVESGGGSVQAGGSLRLSCAASGYTIGPYCMGWFRQAPGKEREGVAAINMGGGIT
YYADSVKGRFTISQDNAKNTVYLLMNSLEPEDTAIYYCAADSTIYASYYECGHGLSTGG
YGYDSWGQGTQVTVSRENLYFQGHHHHHH

Average Mass = 15765

ESI-MS:





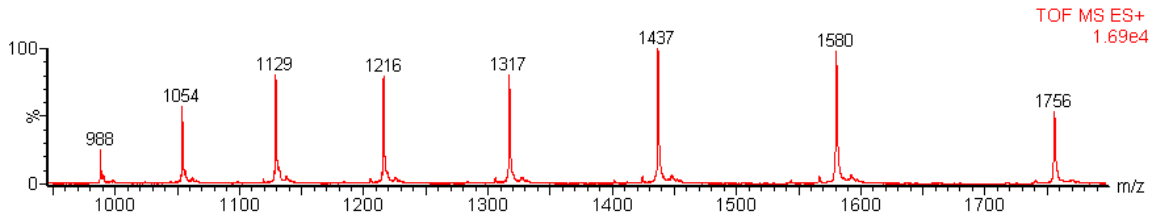
cAb-Lys3-A104C

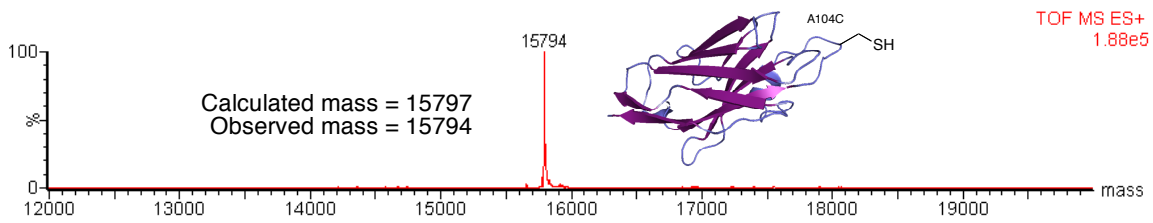
Sequence:

DVQLVESGGGSVQAGGSLRLSCAASGYTIGPYCMGWFRQAPGKEREGVAAINMGGGIT
YYADSVKGRFTISQDNAKNTVYLLMNSLEPEDTAIYYCAADSTIYCSYYECGHGLSTGG
YGYDSWGQGTQVTVSRENLYFQGHHHHHH

Average Mass = 15797

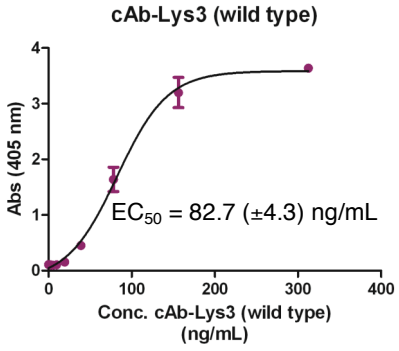
ESI-MS:

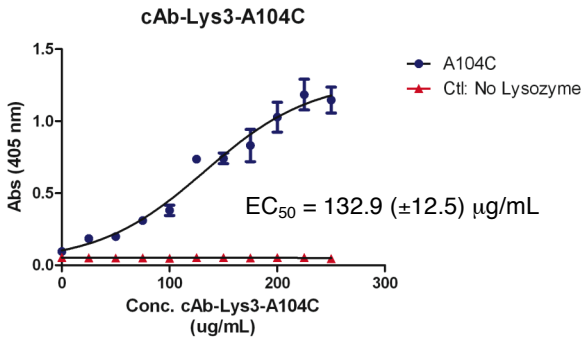




ELISA for cAb-Lys3 and cAb-Lys3-A104C

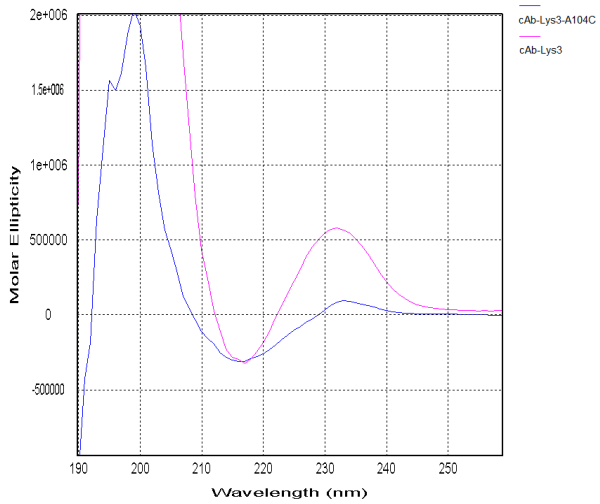
Each well of a 96-well plate (Greiner Bio-one half-area high-binding plates) was coated with 1 μg of hen egg white lysozyme (Sigma), and incubated at 4 °C overnight. All subsequent steps were performed at room temperature. The plate was washed four times with PBS (pH 8.0) containing 5% Tween20 (Sigma), blocked for 90 minutes with 3% BSA in PBS and then washed once with PBS (pH 8.0) containing 5% Tween20. Antibody (cAb-Lys3 (wild type) or cAb-Lys3-A104C) (0.11 mg/mL in 1% BSA, 0.02% Tween20 in PBS) was added and dilutions were made across the plate for final concentrations of 2.5 ng/mL to 150 ng/mL for the wild type and from 25 μg/mL to 250 μg/mL for the A104C mutant. A control sample was also run where no lysozyme was bound to the plate. The plate was incubated with gentle rocking for 70 minutes. Unbound antibody was removed by washing four times with PBS (pH 8.0) containing 5% Tween20. The secondary antibody (Anti-polyHistidine-alkaline phosphatase conjugate, Sigma) was prepared at a 1:1000 dilution in PBS (pH 8.0) and 50 μL was added to each well. The plate was incubated with gentle rocking for 1 hour. Each well was then washed with 50 μL of diethanolamine buffer (Phosphatase substrate kit, Thermo Scientific). PNPP phosphatase substrate (Phosphatase substrate kit, Thermo Scientific) was prepared by dissolving one tablet in 6 mL of diethanolamine buffer. 50 μL of the PNPP substrate solution was added to each well and incubated for 25 min. After this time, absorbance was measured at 405 nm. Absorbance was plotted against concentration. Sigmoidal regression analysis was carried out using GraphPad Prism 5.01. Response curves and EC_{50} values for these conditions are shown below.





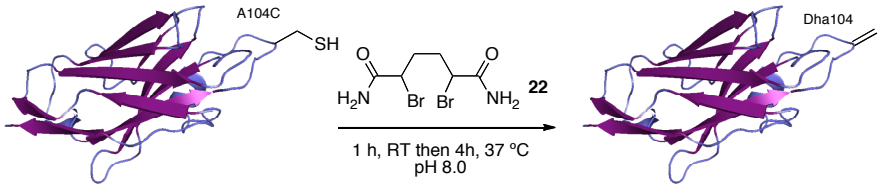
Circular Dichroism

CD measurements were made using a Chirascan spectropolarimeter fitted with a Peltier temperature controller. cAb-Lys3 (wild type) and cAb-Lys3-A104C were each made up as 1.0 mg/mL solutions each in 10 mM PBS at pH 8. CD spectra were measured in a 1 mm quartz cuvette at room temperature using a scan rate of 50 nm/min, 1 nm interval, 1 nm bandwidth and a response time of 0.5 s. After baseline correction, ellipticities in deg were converted to molar ellipticities (deg cm^2 dmol-res^{-1}) by normalizing for the concentration of peptide bonds and path length. The resulting CD spectra for cAb-Lys3 (wild type) and cAb-Lys3-A104C are shown below. The secondary structure components, calculated using CDNN CD spectra deconvolution, are tabulated below for both cAb-Lys3 (wild type) and cAb-Lys3-A104C.



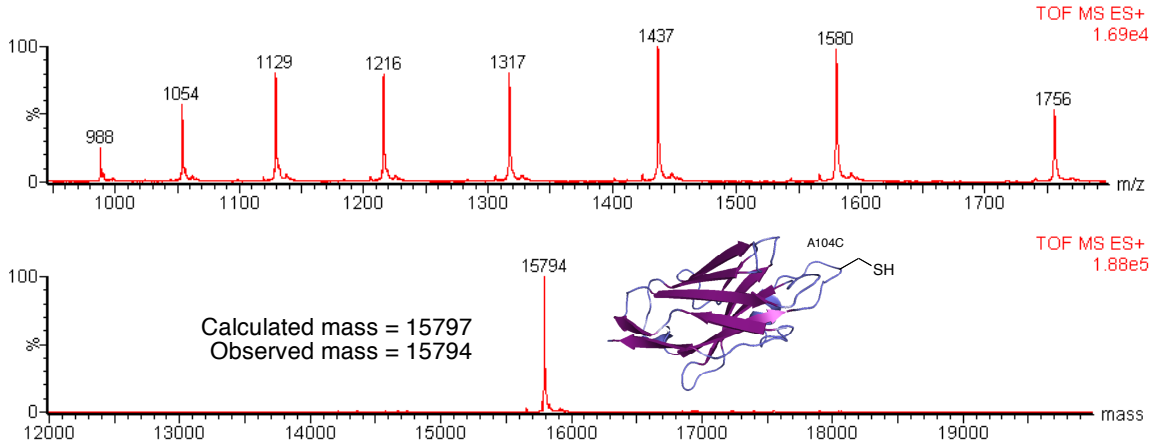
Secondary Structure	Wild Type (%)	A104C (%)
Helix	13.8	11.8
Antiparallel	13.3	13.5
Parallel	13.9	14.8
Beta turn	16.9	16.4
Random coil	42.2	43.5

Dha formation on cAb-Lys3-A104C using reagent 22



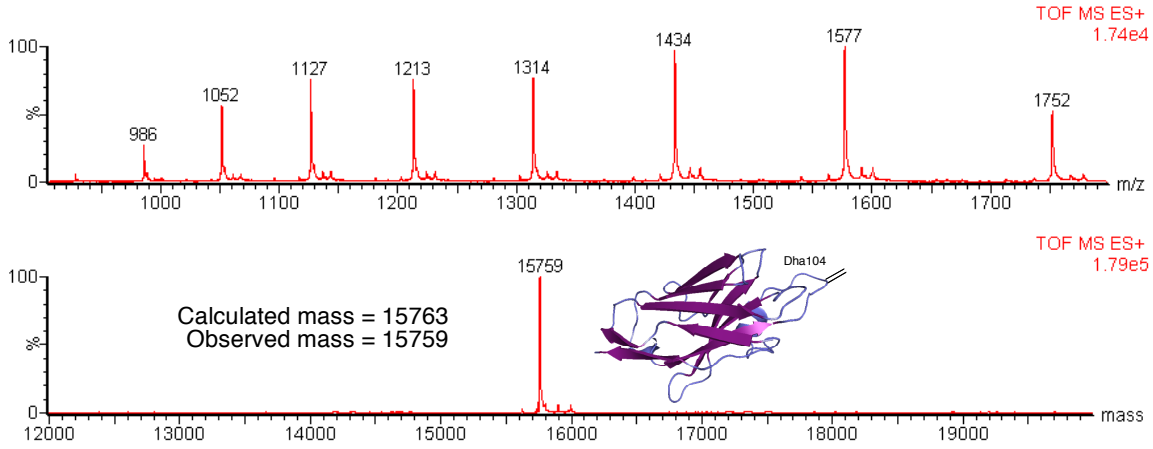
cAb-Lys3-A104C was first reduced with DTT to remove any contaminant disulfide at A104C: DTT (1.2 mg, 7.8 μmol) was added to 500 μL of cAb-Lys3-A104C (c = 1 mg/mL in 10 mM PBS, pH 8.0) and shaken at room temperature for 15 minutes. After this time, the protein solution was

passed through a PD minitrap (GE Healthcare), previously equilibrated with 10 mM PBS (pH 8.0), eluting with 1 mL of the same buffer. The reduced protein was stored on ice until needed. The protein was analyzed by LC-MS, revealing a mass corresponding to the cAb-Lys3-A104C mutant (calculated mass = 15797; observed mass = 15794). ESI-MS are shown below.

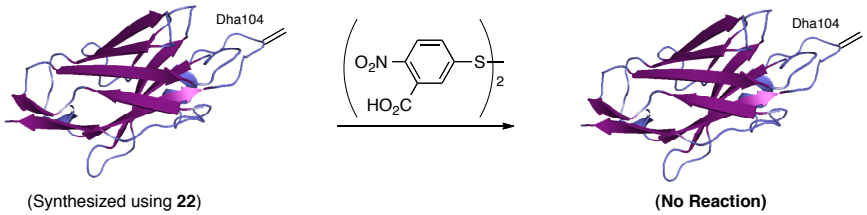


Conversion of Cys104 to Dha104:

Di-bromide **22** (1.2 mg, 3.3 μmol) was added to a 1.5 mL plastic tube as a solid. An 875 μL aliquot of the reduced cAb-Lys3-A104C prepared above (c = 0.5 mg/mL in 10 mM PBS, pH 8.0) was added to the same tube. No DMF was used in this reaction. The reaction was shaken at room temperature for 1 hour, and then 37 °C for 4 hours. The reaction was then cooled to room temperature and precipitated **22** was removed by centrifugation (1 min, 16K g). LC-MS analysis of the supernatant revealed full conversion to a protein with a mass corresponding to the formation of dehydroalanine (calculated mass = 15763; observed mass = 15759). ESI-MS are shown below.

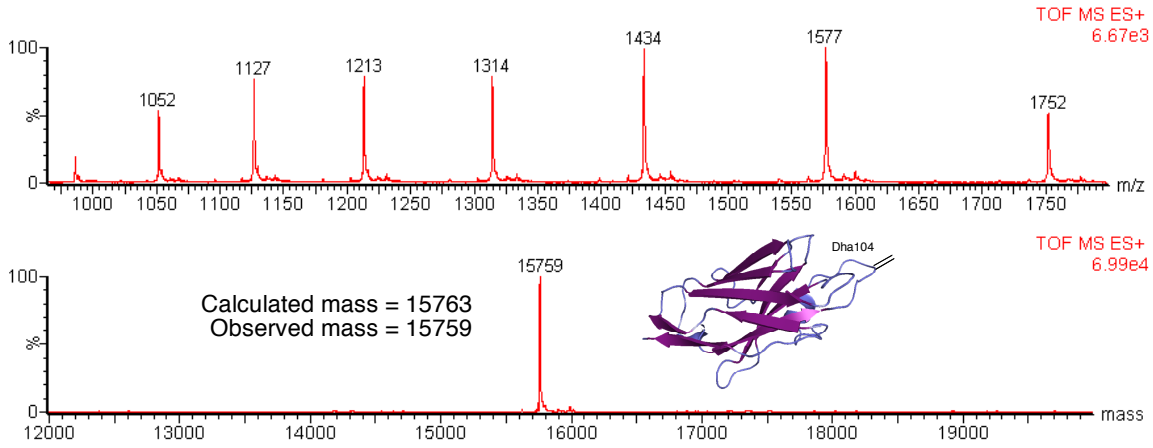


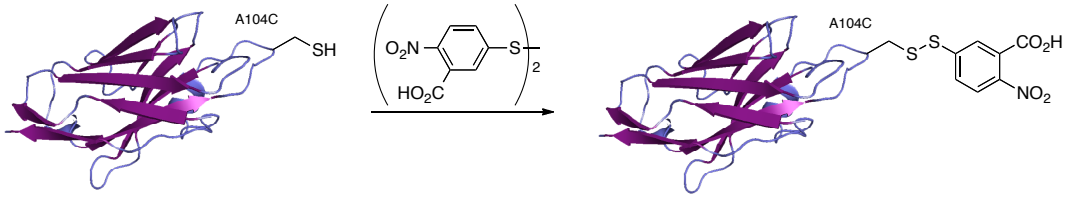
Ellman's Test to confirm all free cysteine consumed after reaction with 22 :



(Synthesized using **22**) **(No Reaction)**

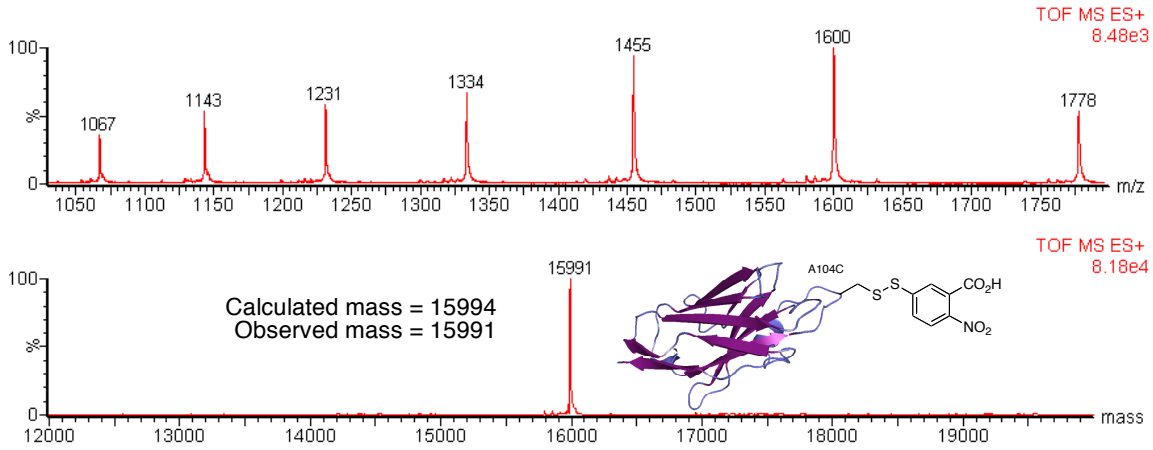
A stock solution of Ellman's reagent was prepared by dissolving 0.6 mg in 108 μL 10 mM PBS, pH 8.0. A 75 μL aliquot cAb-Lys3-104Dha (c = 0.5 mg/mL in 10 mM PBS, pH 8.0, prepared above) was diluted with 75 μL 10 mM PBS, pH 8.0. A 10 μL aliquot of the Ellman's reagent stock solution was added to the diluted protein. The solution was shaken at room temperature for 15 minutes. After this time, the reaction was analysed directly by LC-MS. No reaction was observed, consistent with the complete consumption of cysteine in the elimination reaction (calculated mass = 15763; observed mass = 15759). ESI-MS are shown below. Under identical conditions, unmodified cAb-Lys3-A104C reacts with Ellman's reagent to form the corresponding disulfide (see next).



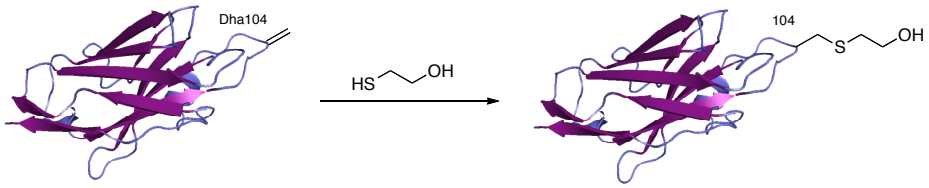


A 75 μL aliquot of cAb-Lys3-A104C (c = 0.5 mg/mL in 10 mM PBS, pH 8.0) was diluted with 75 μL of 10 mM PBS, pH 8.0. A 10 μL aliquot of the Ellman's reagent stock solution was added to the diluted protein. The solution was shaken at room temperature for 15 minutes. After this

time, the reaction was analysed directly by LC-MS. Full conversion to the corresponding disulfide was observed. (calculated mass = 15994; observed mass = 15991). ESI-MS are shown below.

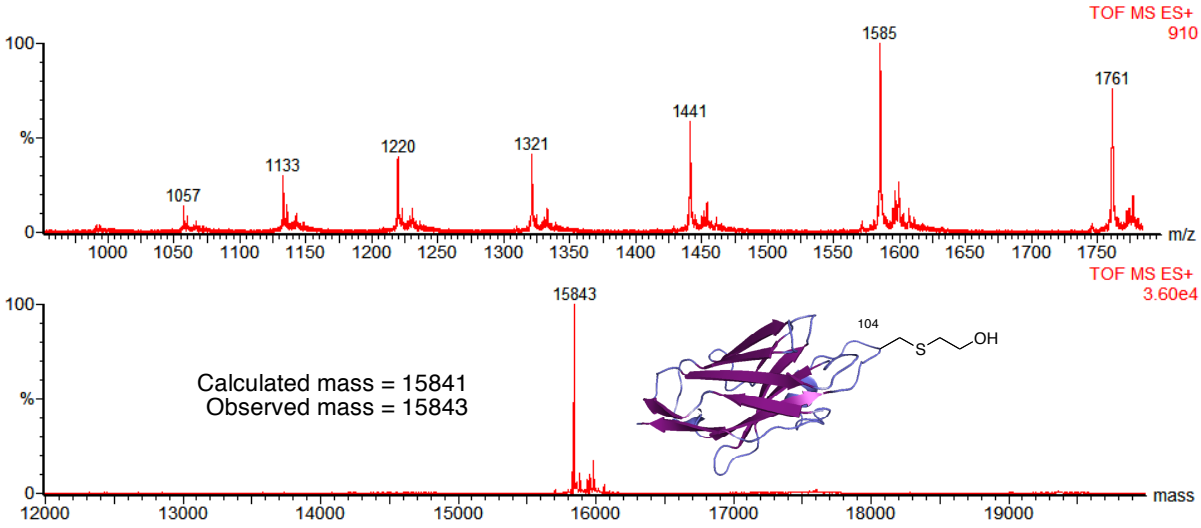


Chemical Test for Dehydroalanine after reaction of cAb-Lys3-A104C with reagent 22: Addition of 2-Mercaptoethanol



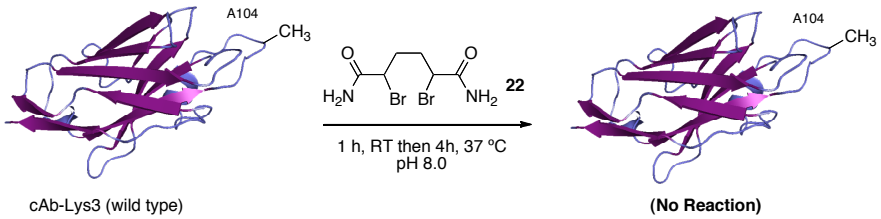
(Synthesized using **22**)

cAb-Lys3-C104Dha was prepared as described above and desalted using a PD minitrap. To 250 µL of desalted protein (c = 0.25 mg/mL in 10 mM PBS, pH 8.0) was added 2-mercaptoethanol (5 µL), and the reaction shaken for 1 hour at 37 °C. A mass corresponding to the 2-mercaptoethanol adduct was observed by LC-MS (calculated mass = 15841, observed mass = 15843). ESI-MS are shown below.



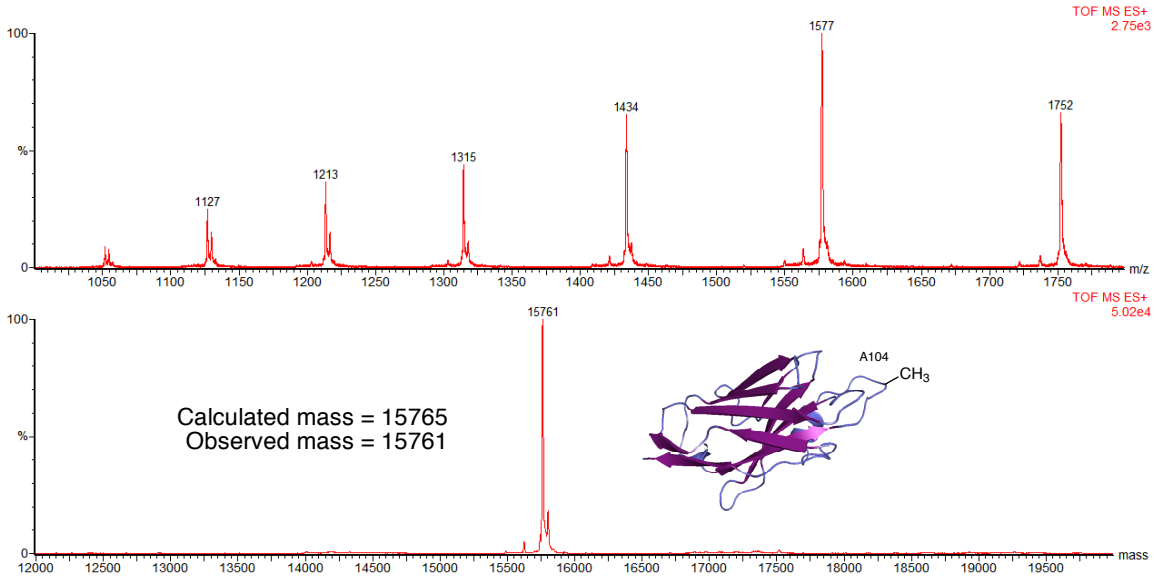
Control experiments with cAb-Lys3 (wild type)

cAb-Lys3 (wild type) does not react with reagent 22

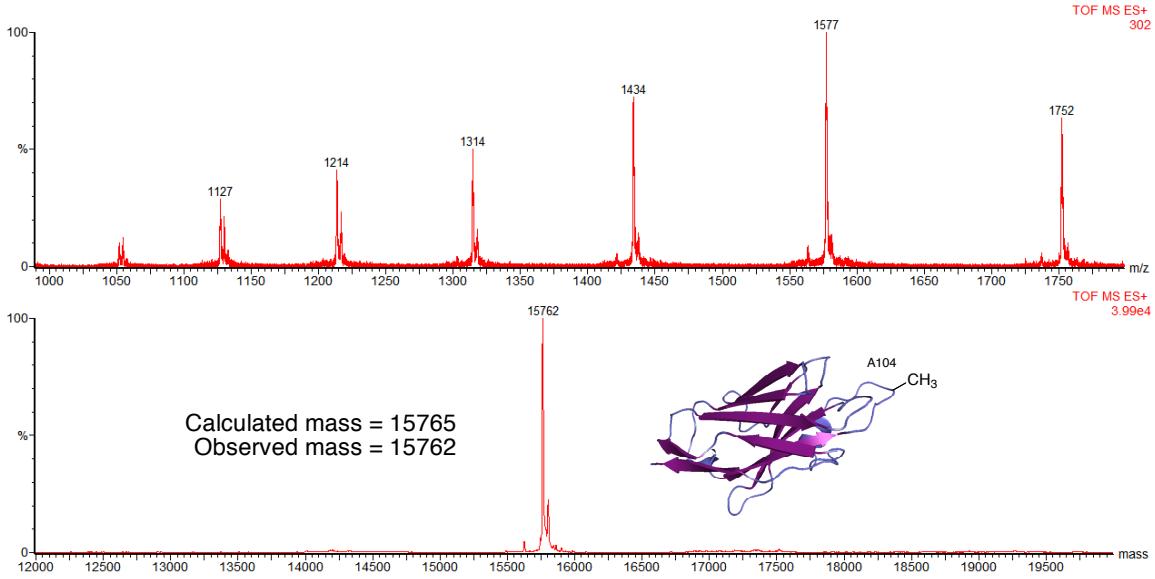


cAb-Lys3 (wild type) **(No Reaction)**

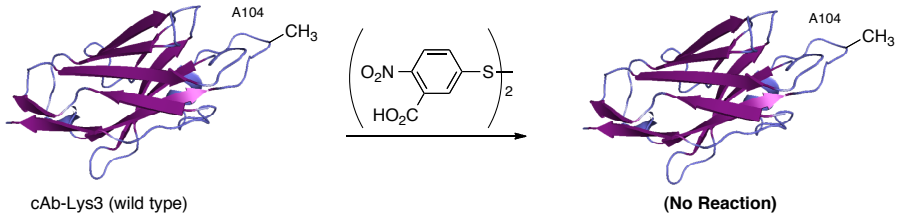
cAb-Lys3 (wild type) was subjected to reaction conditions identical to those used to install dehydroalanine on the A104C mutant. Accordingly, DTT (1 mg, 6.5 μmol) was added to 500 μL of cAb-Lys3 (wild type) (c = 1 mg/mL in 10 mM PBS, pH 8.0) and shaken at room temperature for 15 minutes. After this time, the protein solution was passed through a PD minitrap (GE Healthcare), previously equilibrated with 10 mM PBS (pH 8.0), eluting with 1 mL of the same buffer. The protein was used immediately in the next step. The protein was analyzed by LC-MS, revealing a mass corresponding to cAb-Lys3 (wild type) (calculated mass = 15765; observed mass = 15761). ESI-MS are shown below.



Di-bromide **22** (1.2 mg, 3.3 μmol) was added to a 1.5 mL plastic tube as a solid. An 875 μL aliquot of cAb-Lys3 (wild type) previously treated with DTT (c = 0.5 mg/mL in 10 mM PBS, pH 8.0) was added to the same tube. No DMF was used in this reaction. The reaction was shaken at room temperature for 1 hour, and then 37 °C for 4 hours. The reaction was then cooled to room temperature and precipitated **22** was removed by centrifugation (1 min, 16K g). LC-MS analysis of the supernatant revealed a mass corresponding to unmodified cAb-Lys3 (wild type) (calculated mass = 15765; observed mass = 15762), indicating cAb-Lys3 (wild type) did not react with DTT or dibromide **22**. ESI-MS are shown below.

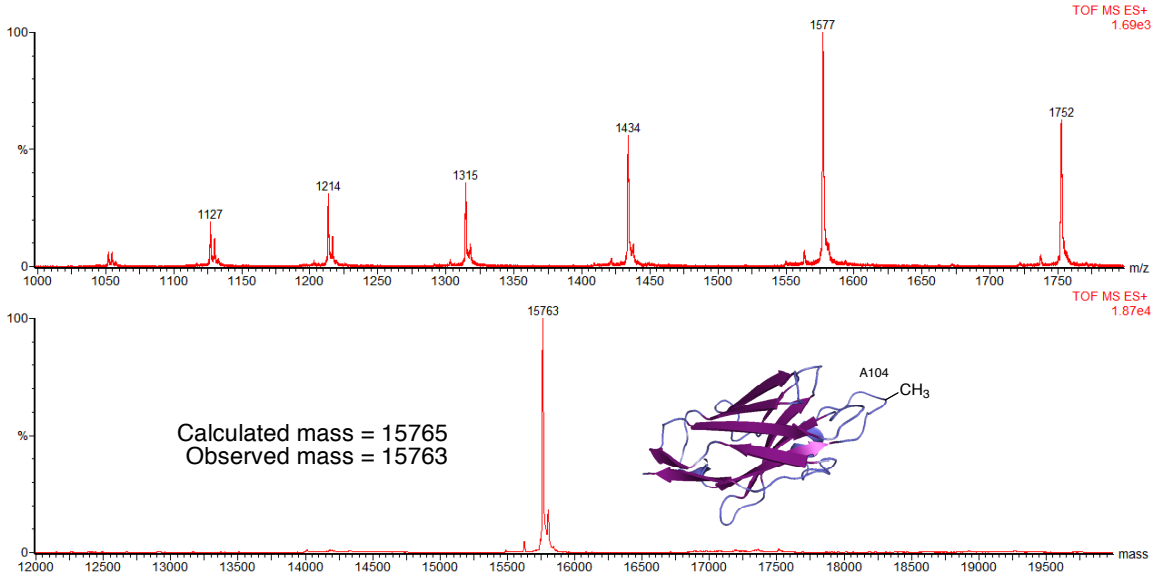


Ellman's test with cAb-Lys3 (wild type)

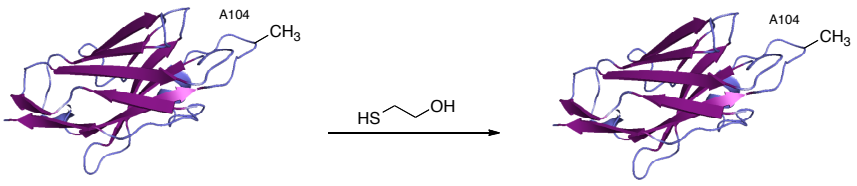


cAb-Lys3 (wild type) **(No Reaction)**

cAb-Lys3 (wild type) was treated with DTT and then desalted using a PD minitrap as described above. A 75 µL aliquot of the resulting protein solution was diluted with 75 µL of 10 mM PBS, pH 8.0. A 10 µL aliquot of the Ellman's reagent stock solution prepared above was added to the diluted protein. The solution was shaken at room temperature for 15 minutes. After this time, the reaction was analysed directly by LC-MS. No reaction was observed, indicating that DTT and Ellman's reagent do not react with cAb-Lys3 (wild type). (calculated mass = 15765; observed mass = 15763). ESI-MS are shown below.

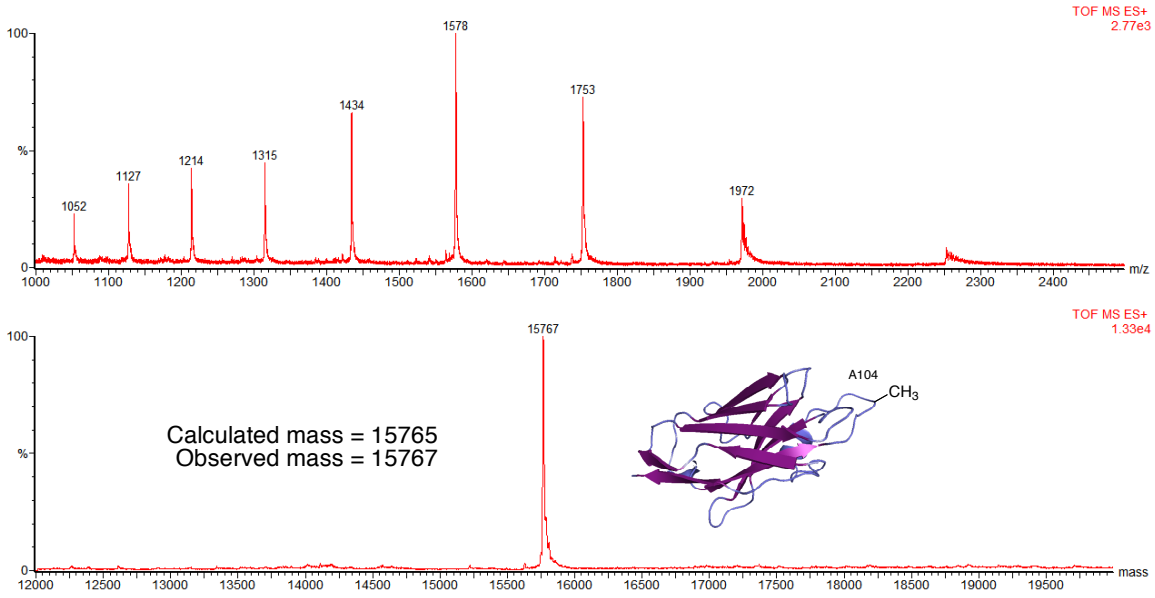


cAb-Lys3 (wild type) does not react with 2-mercaptoethanol

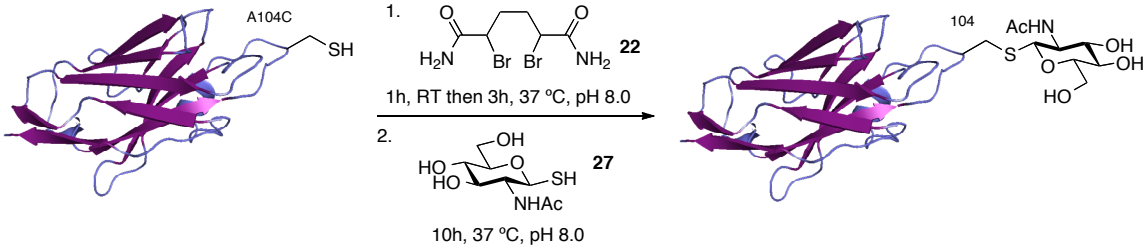


cAb-Lys3 (wild type) **(No Reaction)**

2-Mercaptoethanol (5 µL) was added to 250 µL of cAb-Lys3 (wild type) (c = 0.25 mg/mL in 10 mM PBS, pH 8.0). The solution was shaken for 1 hour at 37 °C. No reaction was observed by LC-MS (calculated mass = 15765, observed mass = 15767). ESI-MS are shown below.



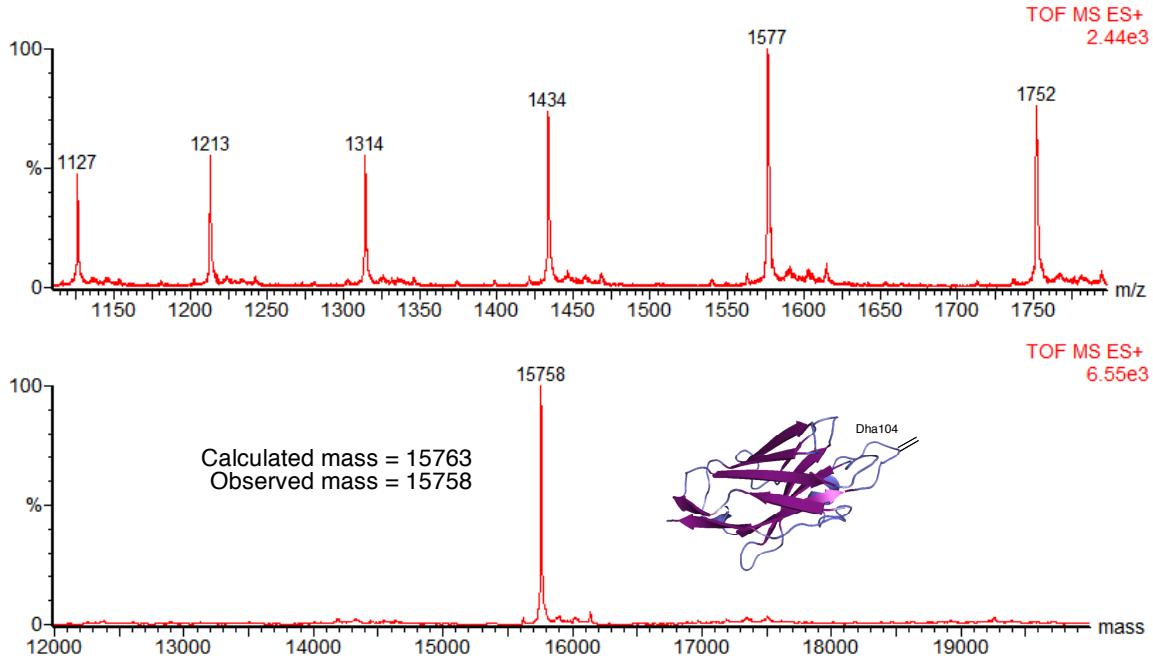
Synthesis of glycosylated cAb-Lys3



cAb-Lys3-A104C was reduced with DTT and passed through a PD minitrap as described above. Di-bromide **22** (1.0 mg, 3.3 μmol) was added to a 1.5 mL plastic tube. A 950 μL aliquot of cAb-Lys3-A104C (c = 0.5 mg/mL in 10 mM PBS, pH 8.0) was added to the same tube. No DMF was used in this reaction. The reaction was shaken at room temperature for 1 hour, and then 37 °C for 3 hours. The reaction was then cooled to room temperature and precipitated **22** was removed by centrifugation (1 min, 16K g). LC-MS analysis of the supernatant revealed full conversion to a protein with a mass corresponding to the formation of dehydroalanine (calculated mass = 15763; observed mass = 15758). This protein solution was used directly for the conjugate addition of GlcNAc thiol **27**. Accordingly, GlcNAc thiol **27** (15 mg, 63 μmol) was added as a solid and the reaction was incubated at 37 °C. Additional **27** was added every 15 minutes until a total of 150 mg was in the reaction mixture. The reaction was monitored directly by LC-MS. Full conversion

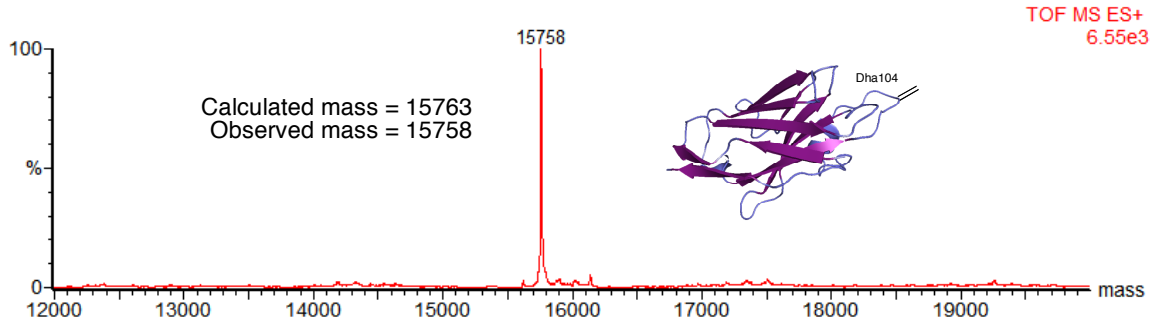
to the glycosylated antibody fragment was observed after 10 hours at 37 ºC (calculated mass = 16000; observed mass = 15996). ESI-MS for both Dha formation and addition of GlcNAc **27** are shown below.

Dha formation after reaction with 22:

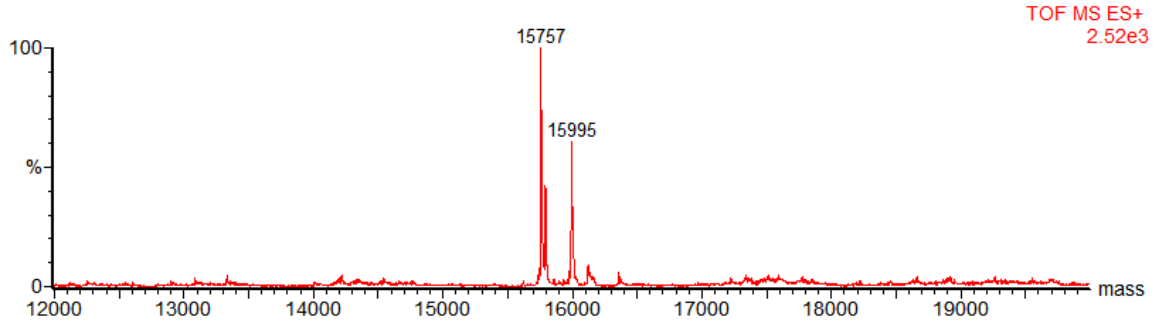


Addition of GlcNAc thiol 27 to Dha at position 104 in cAb-Lys3:

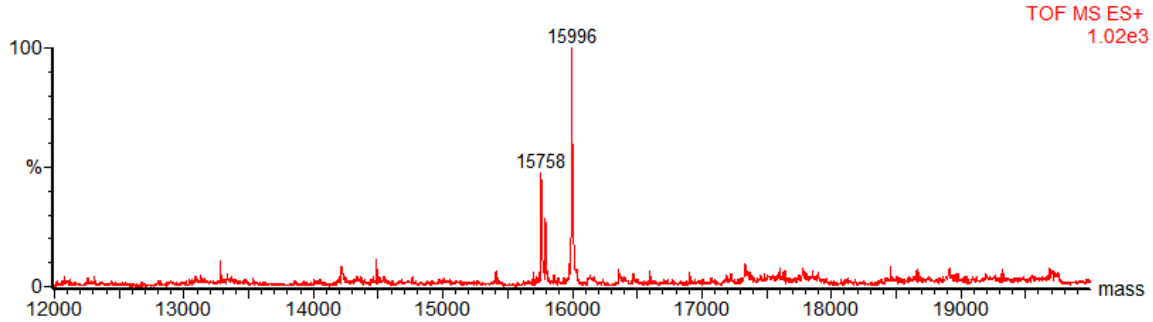
T = 0 (Dha)



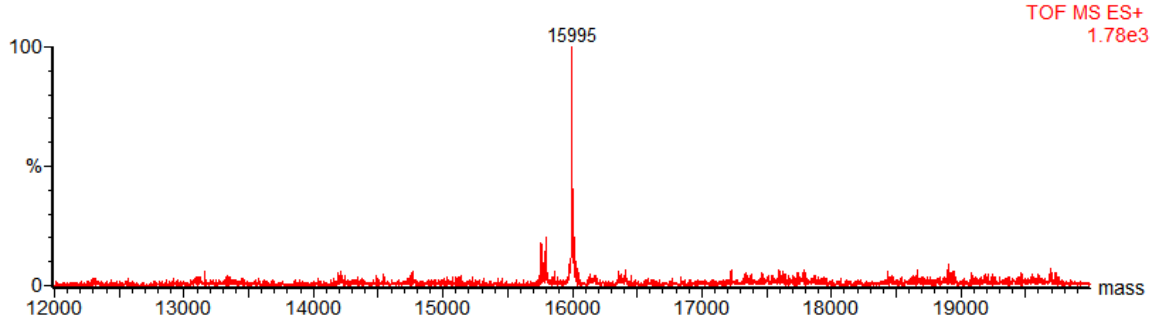
T = 1h



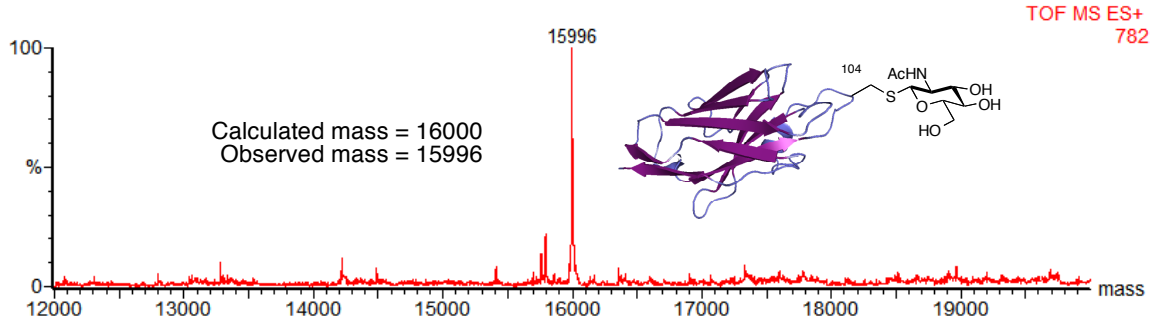
T = 4h



T = 7h



T = 10h



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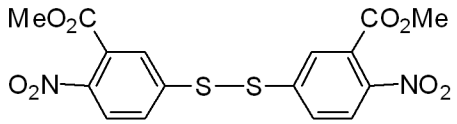

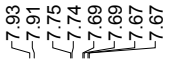

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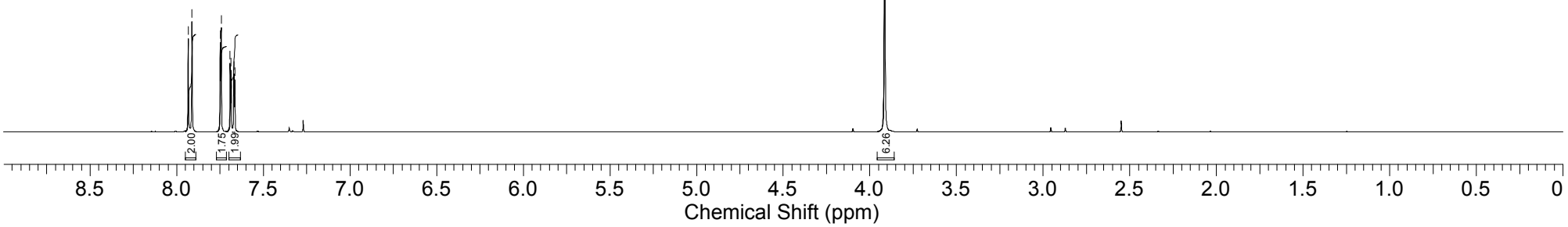

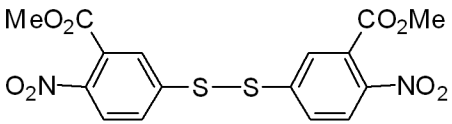

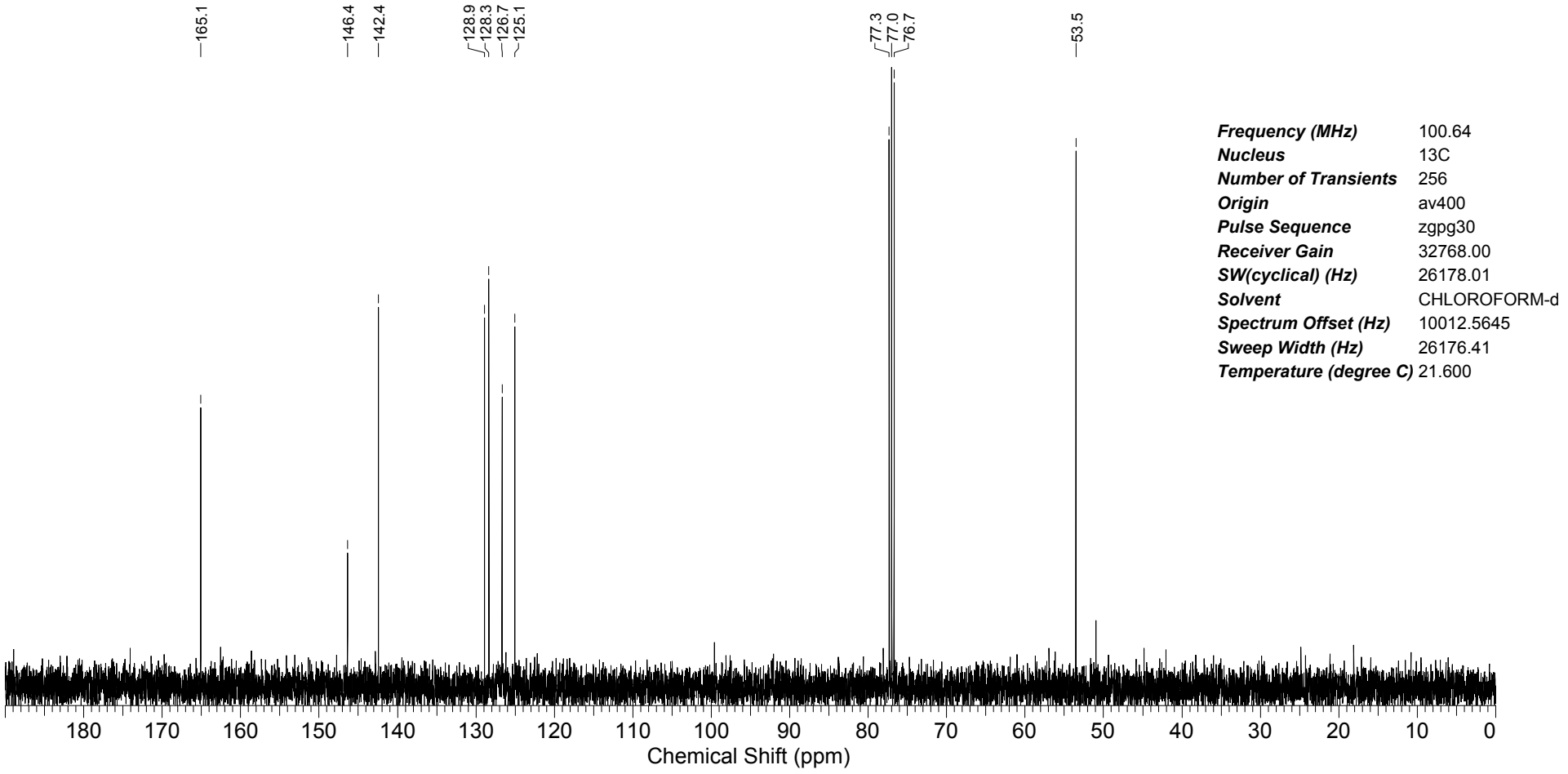

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Nucleus	13C
Number of Transients	256
Origin	av400
Pulse Sequence	zgpg30
Receiver Gain	32768.00
SW(cyclical) (Hz)	26178.01
Solvent	CHLOROFORM-d
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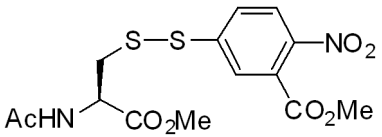

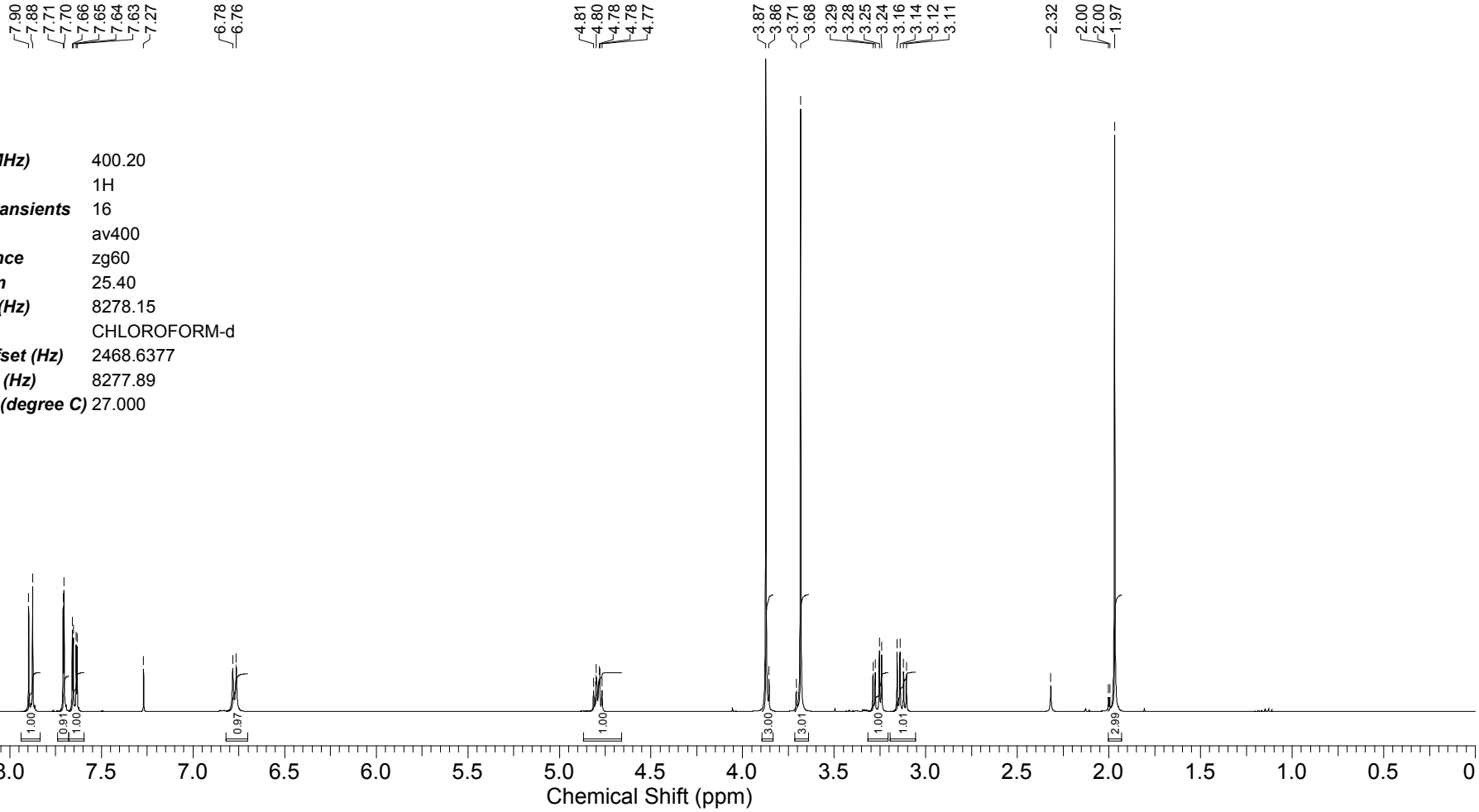

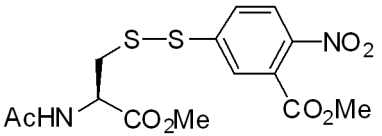

170.3
170.0
165.5
145.3
144.8
128.6
127.9
126.0
124.6
77.3
77.0
76.7
53.3
52.6
51.4
40.4
22.7

Frequency (MHz)	100.64
Nucleus	13C
Number of Transients	256
Origin	av400
Pulse Sequence	zgpg30
Receiver Gain	32768.00
SW(cyclical) (Hz)	26178.01
Solvent	CHLOROFORM-d
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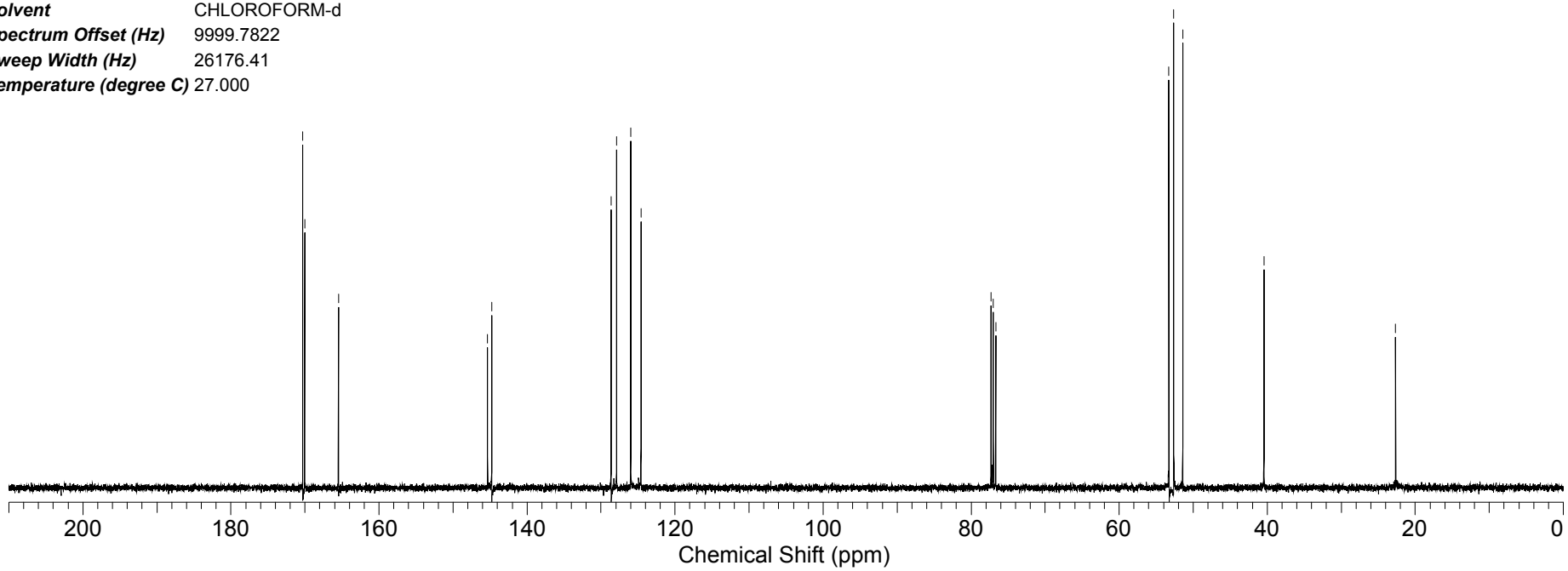
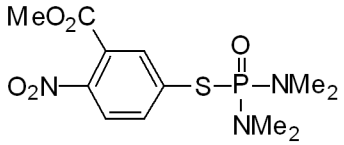
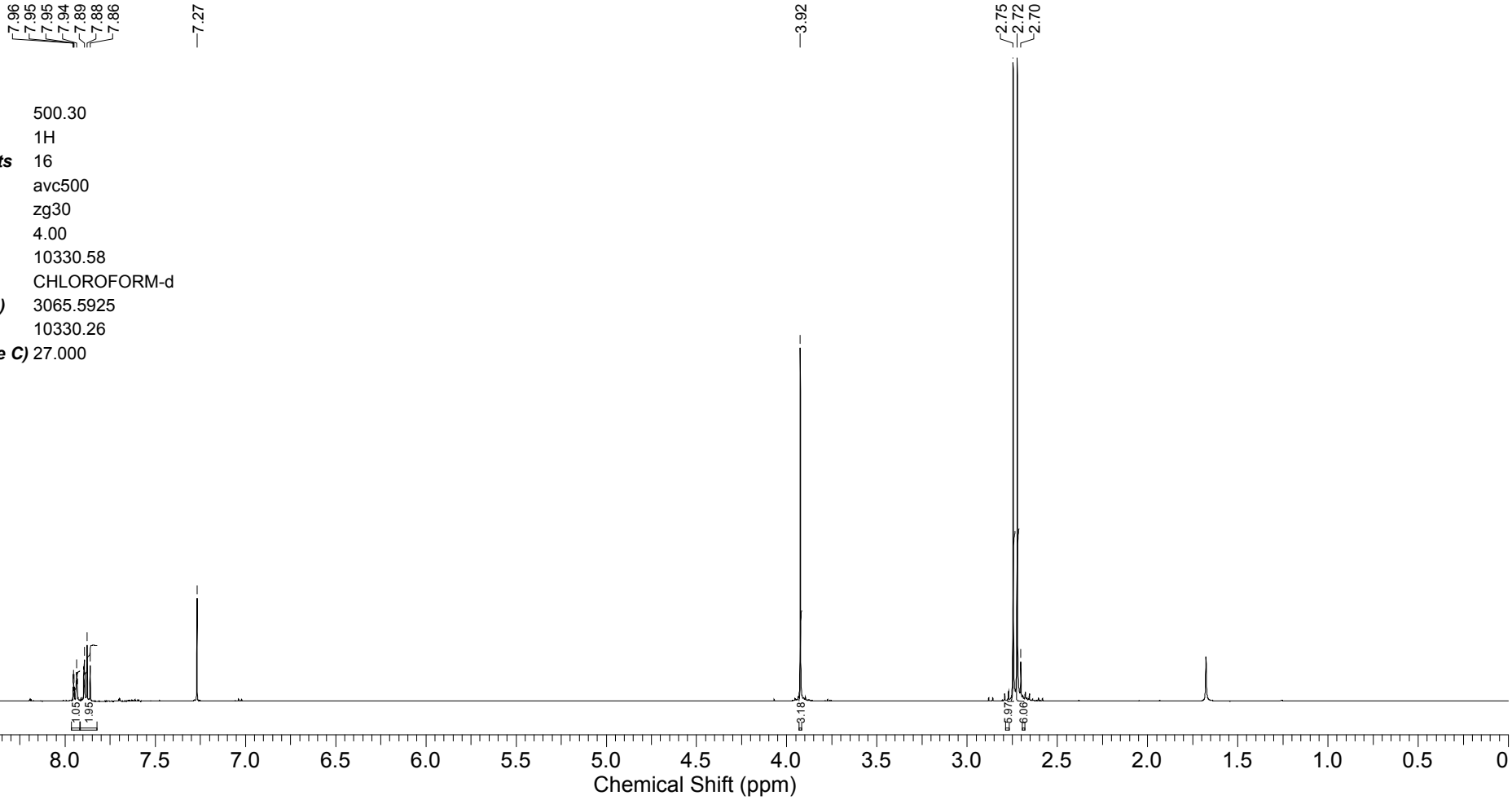
Temperature (degree C)	27.000







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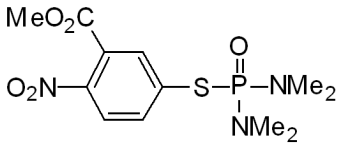

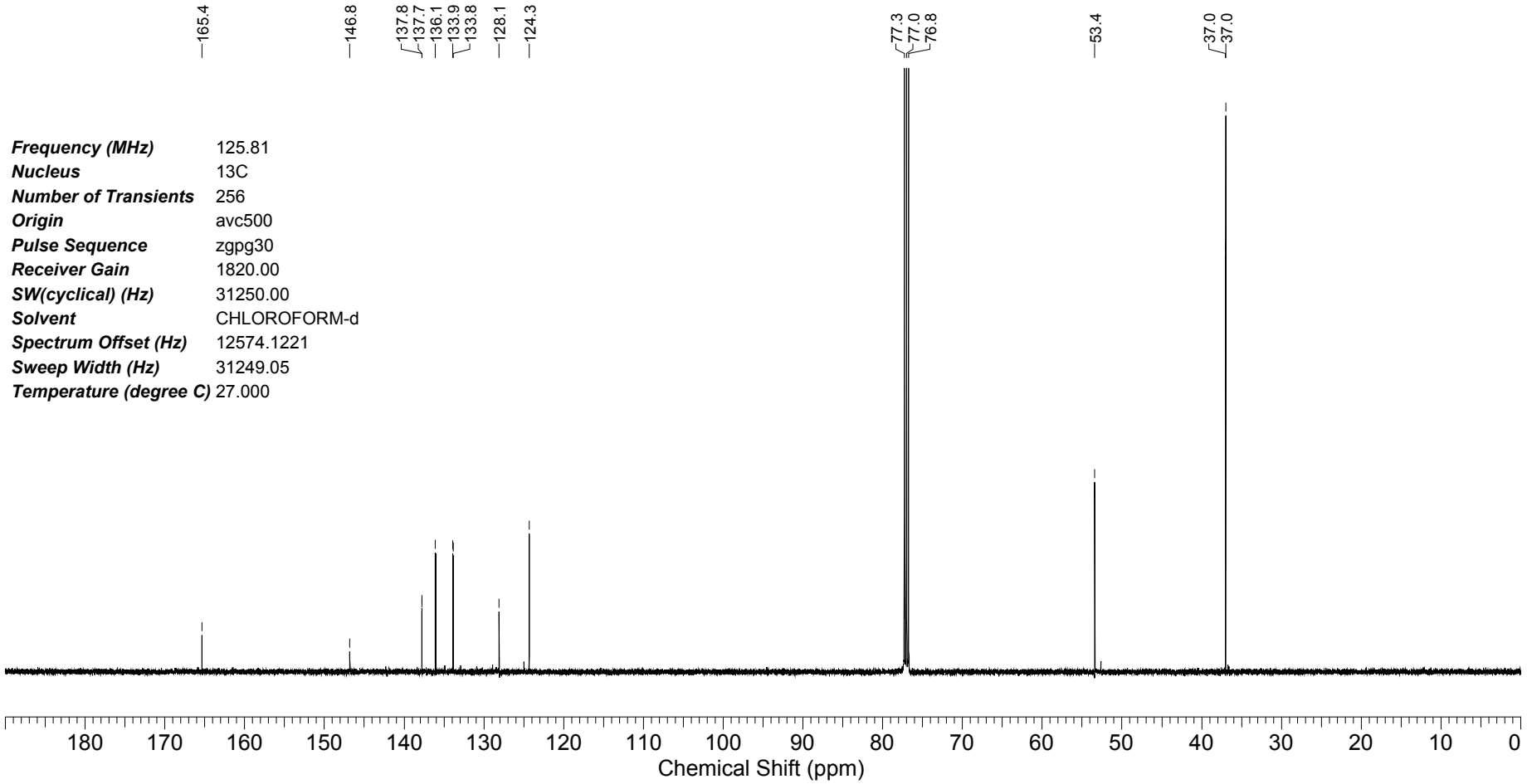

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Number of Transients	256
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Pulse Sequence	zgpg30
Receiver Gain	1820.00
SW(cyclical) (Hz)	31250.00
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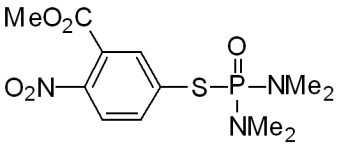
Temperature (degree C)	27.000



137.8
137.7
136.1
136.1
133.9
133.8

Frequency (MHz)	125.81
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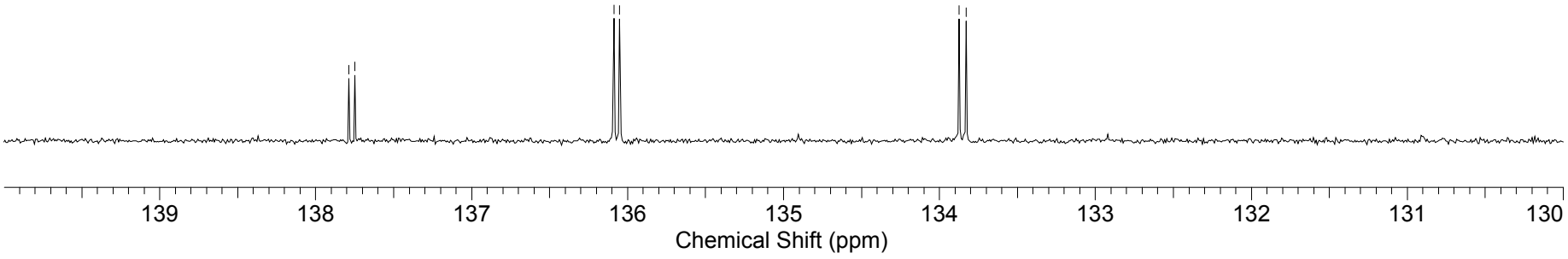

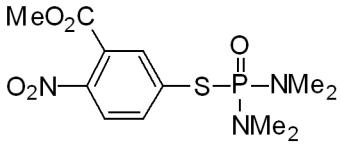

37.6

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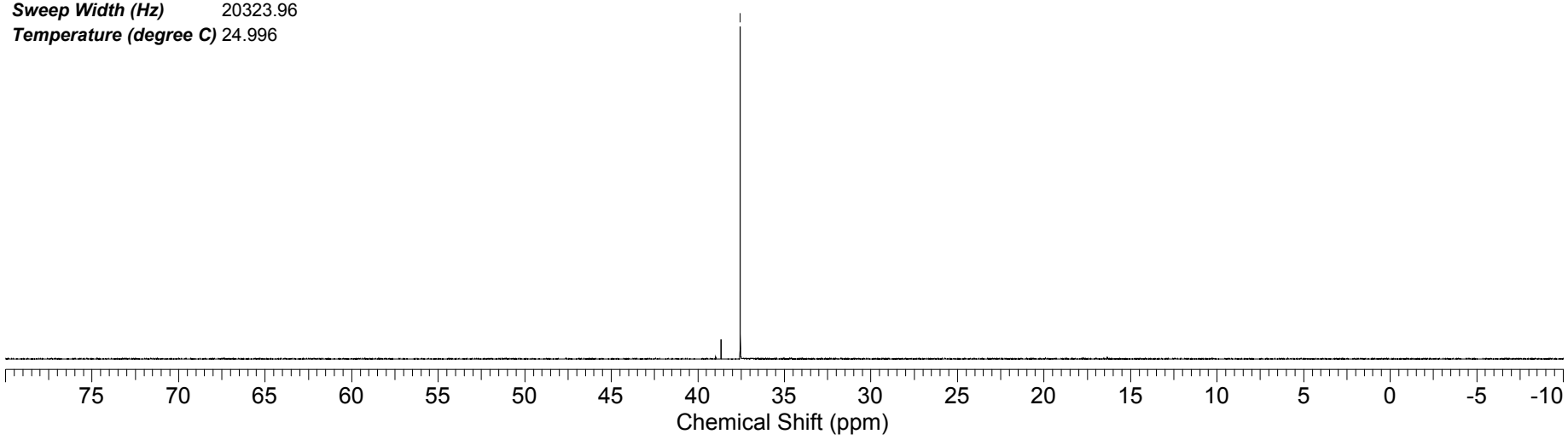
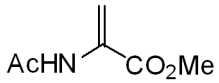
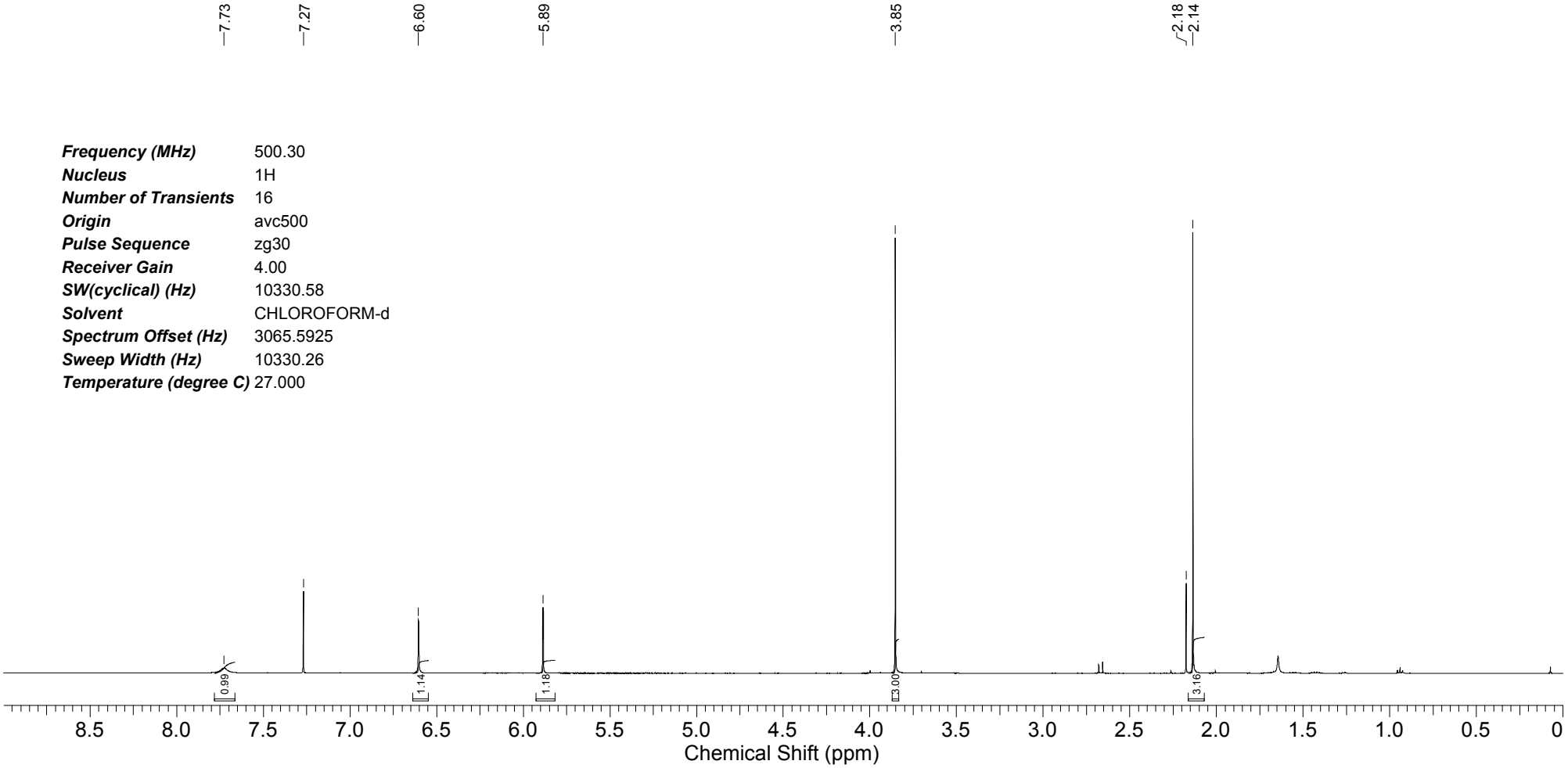
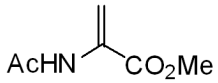
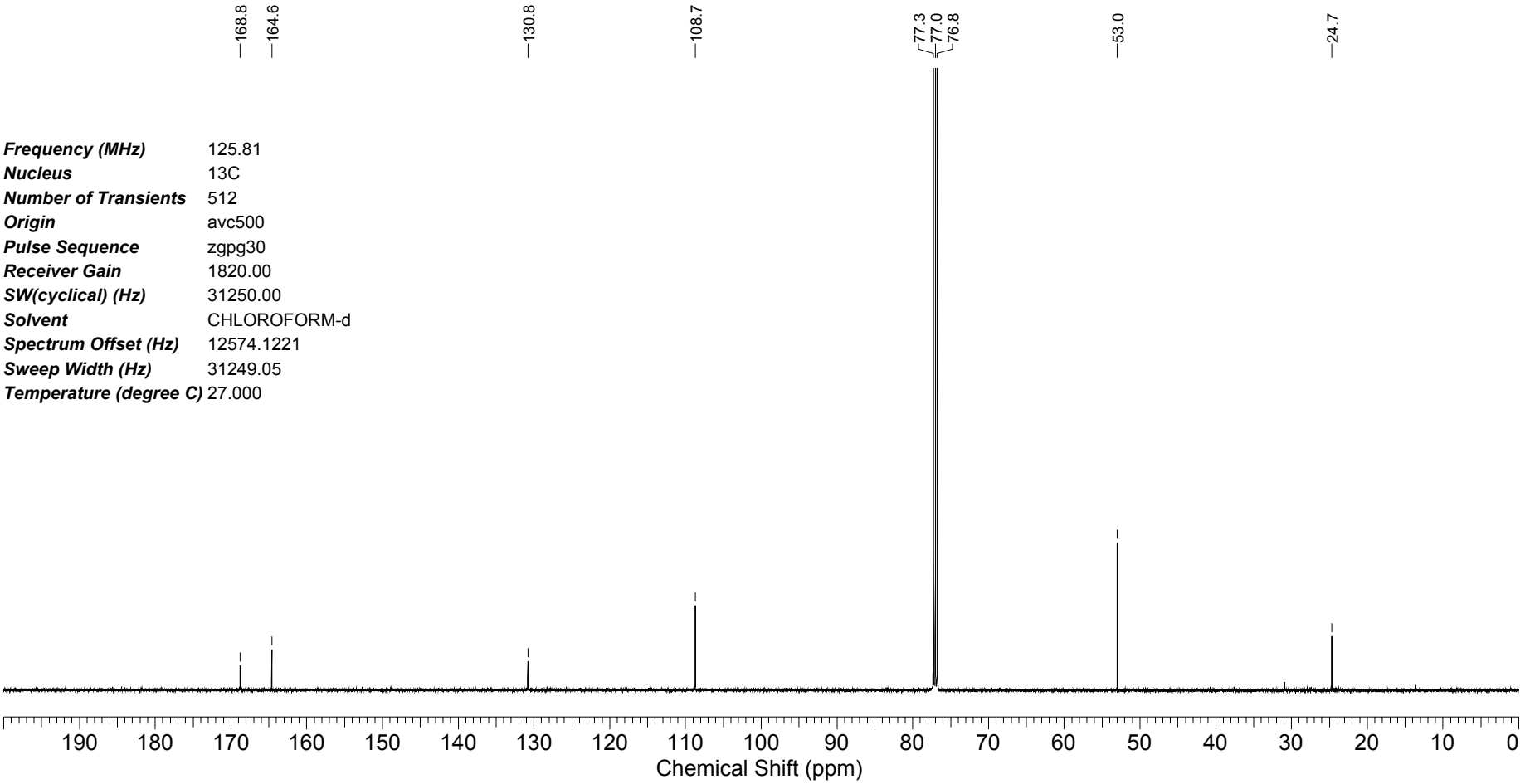


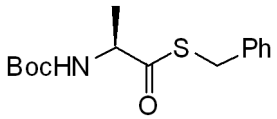




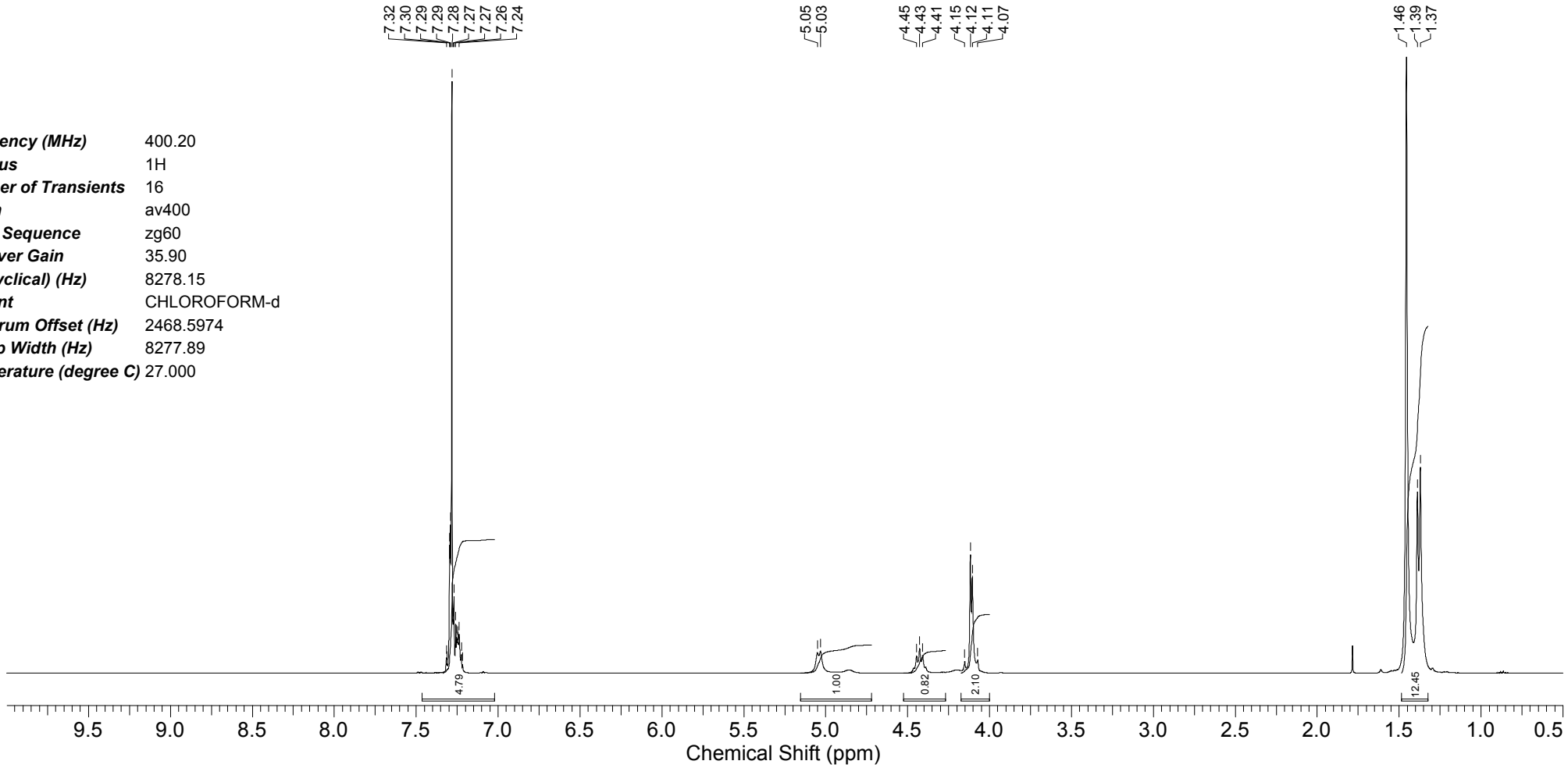


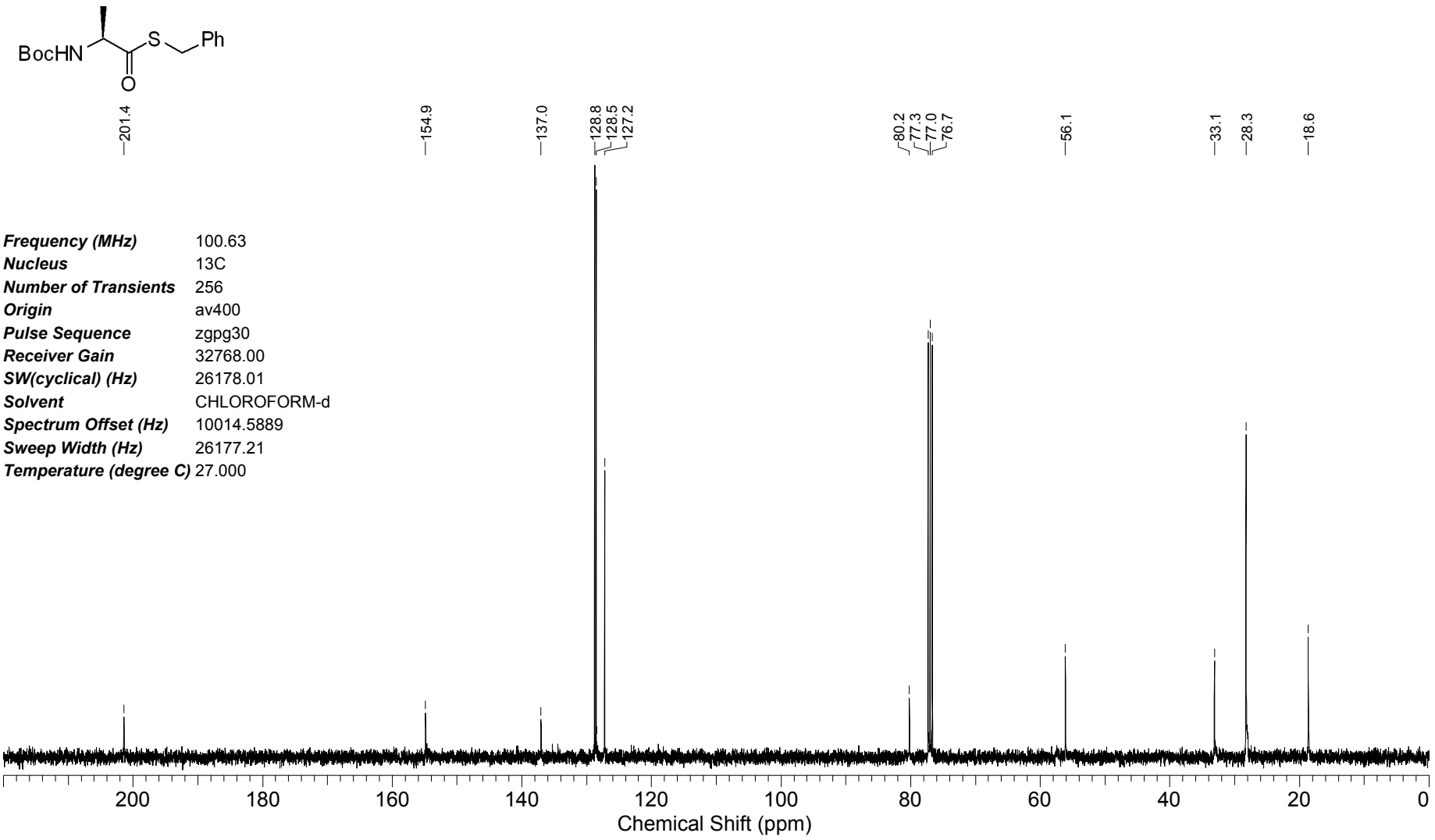


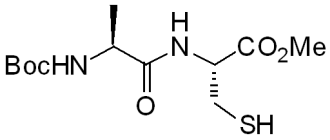
Frequency (MHz)	125.81
Nucleus	13C
Number of Transients	512
Origin	avc500
Pulse Sequence	zgpg30
Receiver Gain	1820.00
SW(cyclical) (Hz)	31250.00
Solvent	CHLOROFORM-d
Spectrum Offset (Hz)	12574.1221
Sweep Width (Hz)	31249.05
Temperature (degree C)	27.000



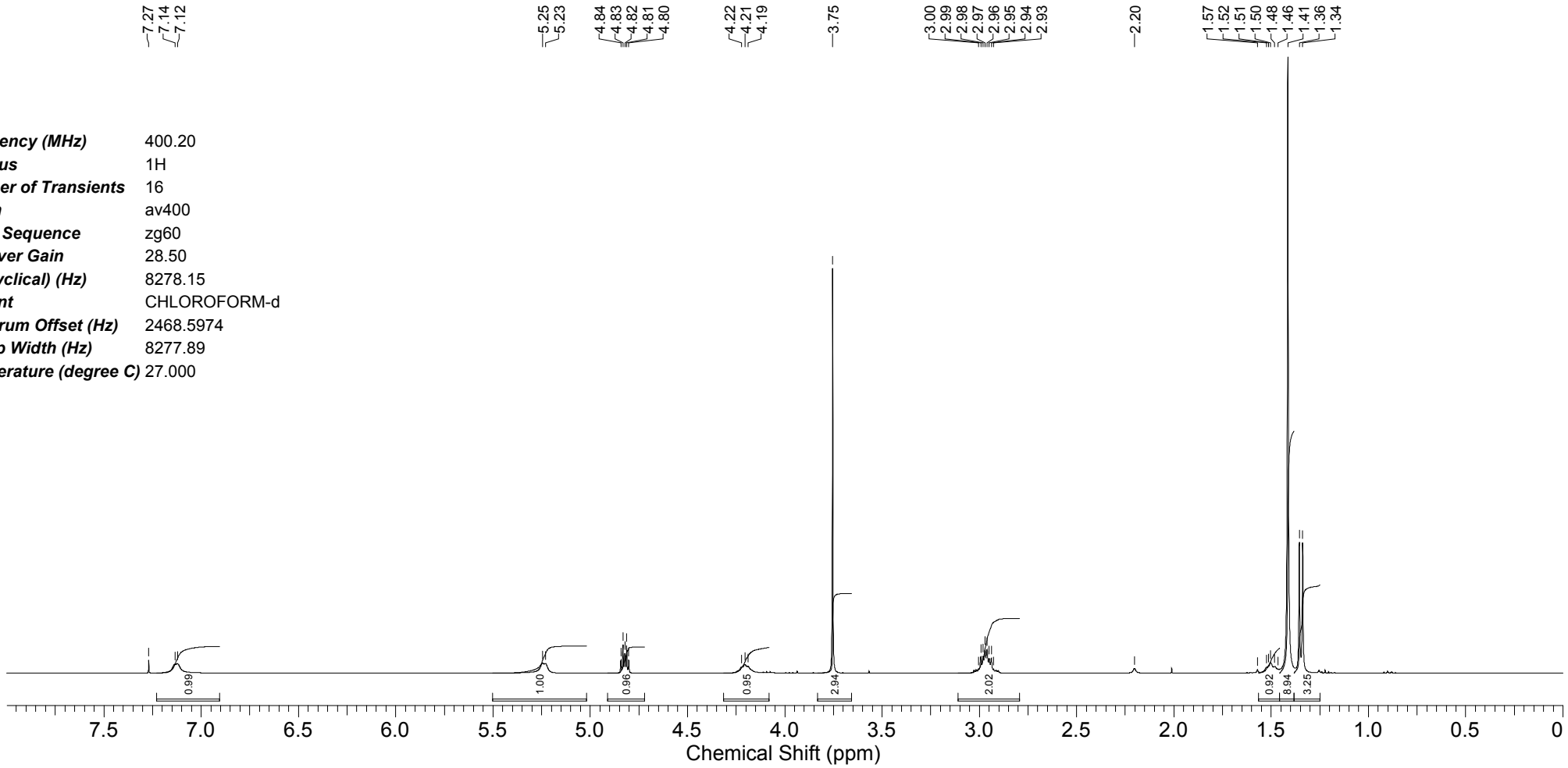
Frequency (MHz)	400.20
Nucleus	1H
Number of Transients	16
Origin	av400
Pulse Sequence	zg60
Receiver Gain	35.90
SW(cyclical) (Hz)	8278.15
Solvent	CHLOROFORM-d
Spectrum Offset (Hz)	2468.5974
Sweep Width (Hz)	8277.89
Temperature (degree C)	27.000

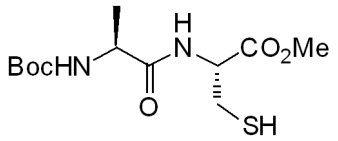


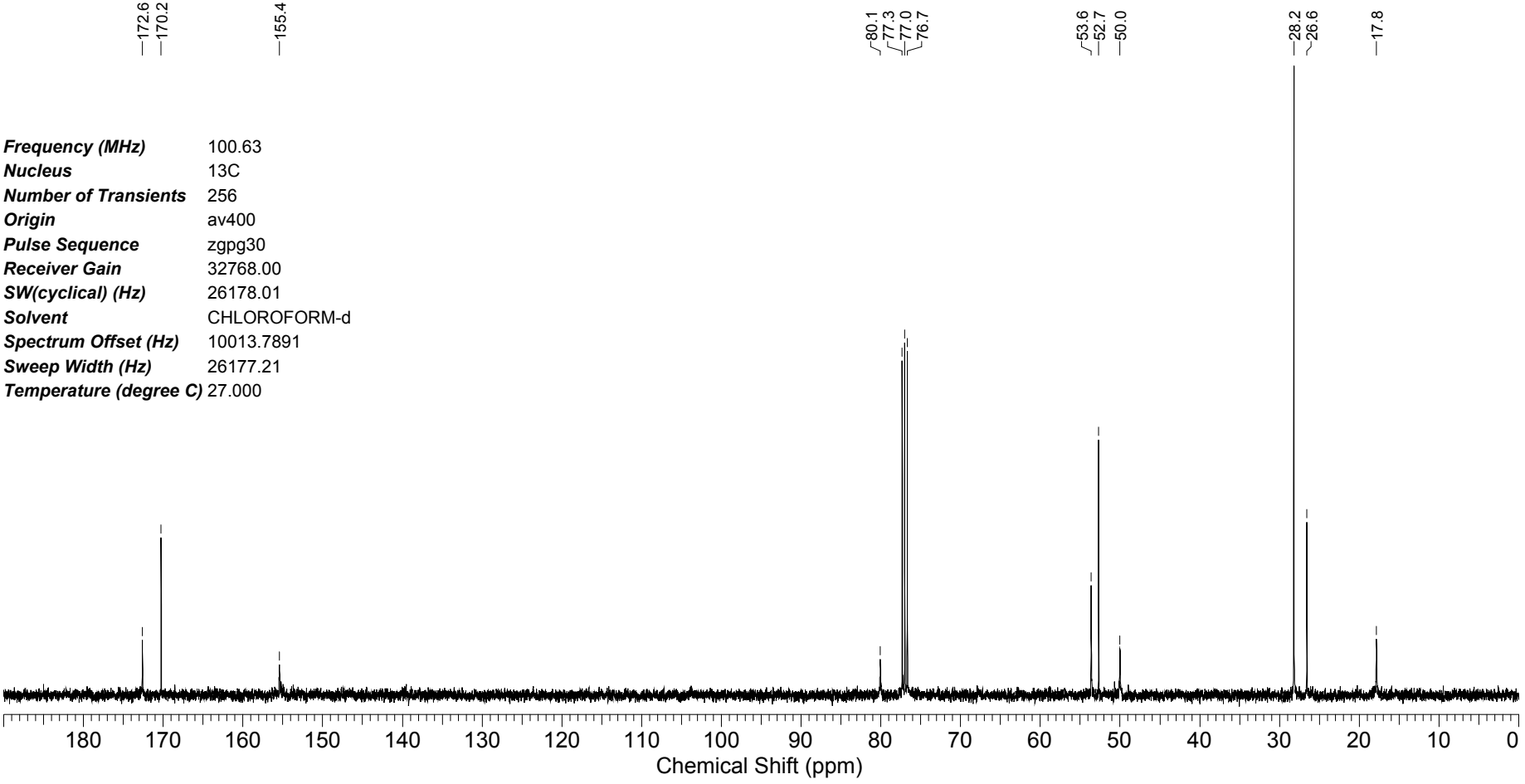




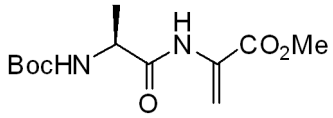
Frequency (MHz)	400.20
Nucleus	1H
Number of Transients	16
Origin	av400
Pulse Sequence	zg60
Receiver Gain	28.50
SW(cyclical) (Hz)	8278.15
Solvent	CHLOROFORM-d
Spectrum Offset (Hz)	2468.5974
Sweep Width (Hz)	8277.89
Temperature (degree C)	27.000

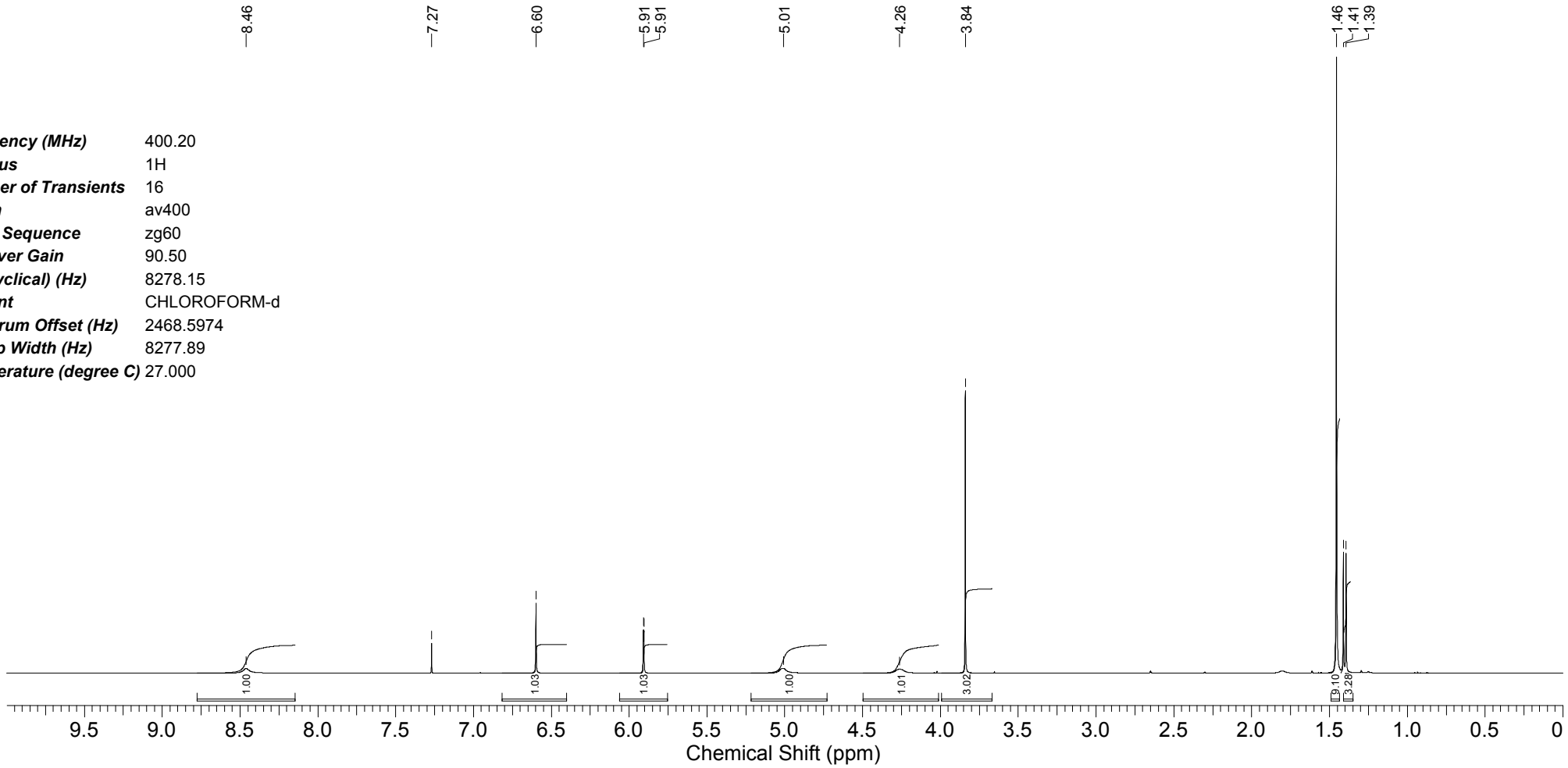




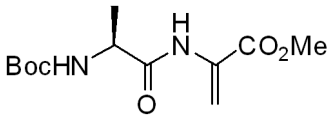


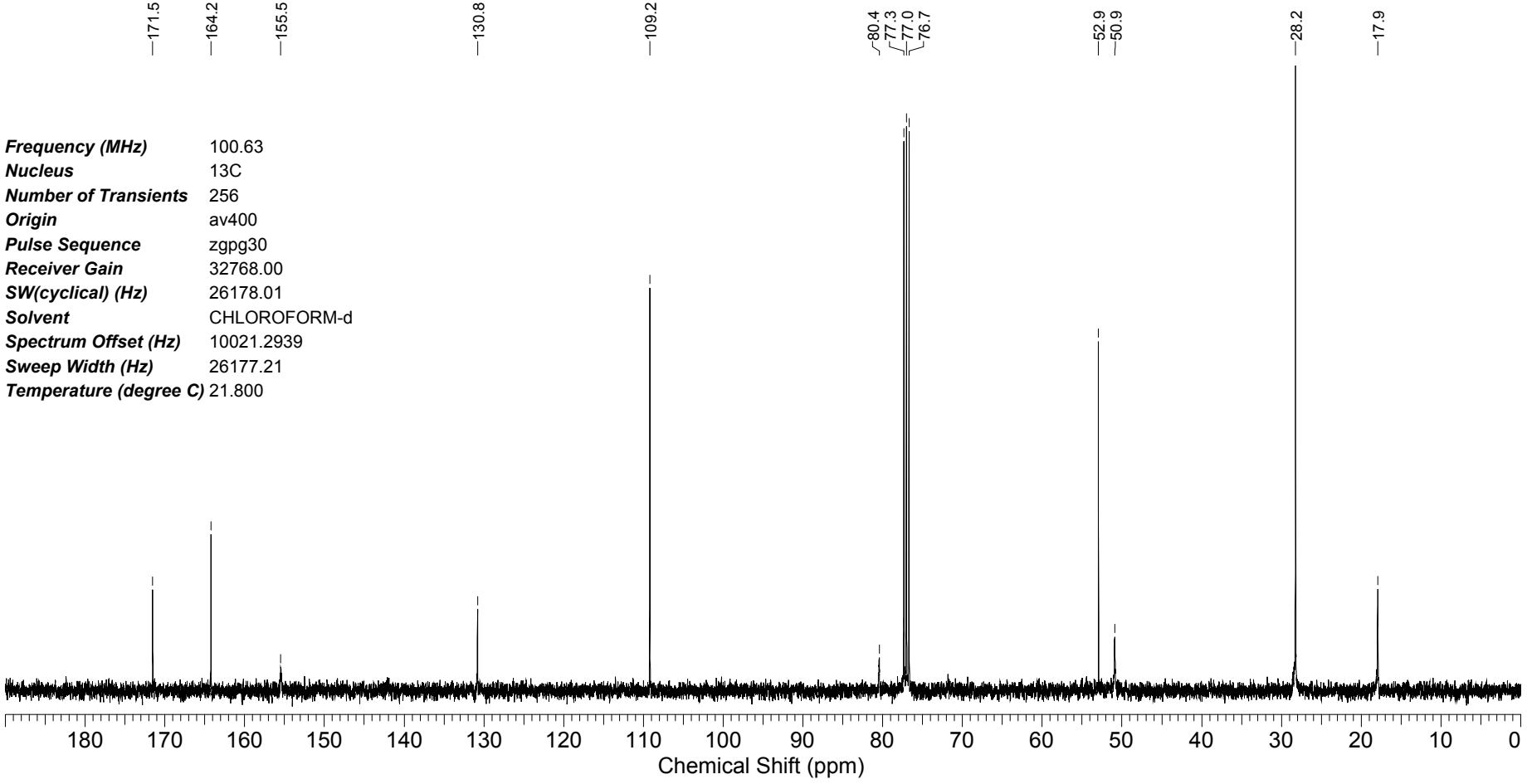
Frequency (MHz)	100.63
Nucleus	13C
Number of Transients	256
Origin	av400
Pulse Sequence	zgpg30
Receiver Gain	32768.00
SW(cyclical) (Hz)	26178.01
Solvent	CHLOROFORM-d
Spectrum Offset (Hz)	10013.7891
Sweep Width (Hz)	26177.21
Temperature (degree C)	27.000

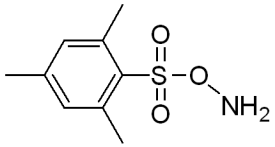


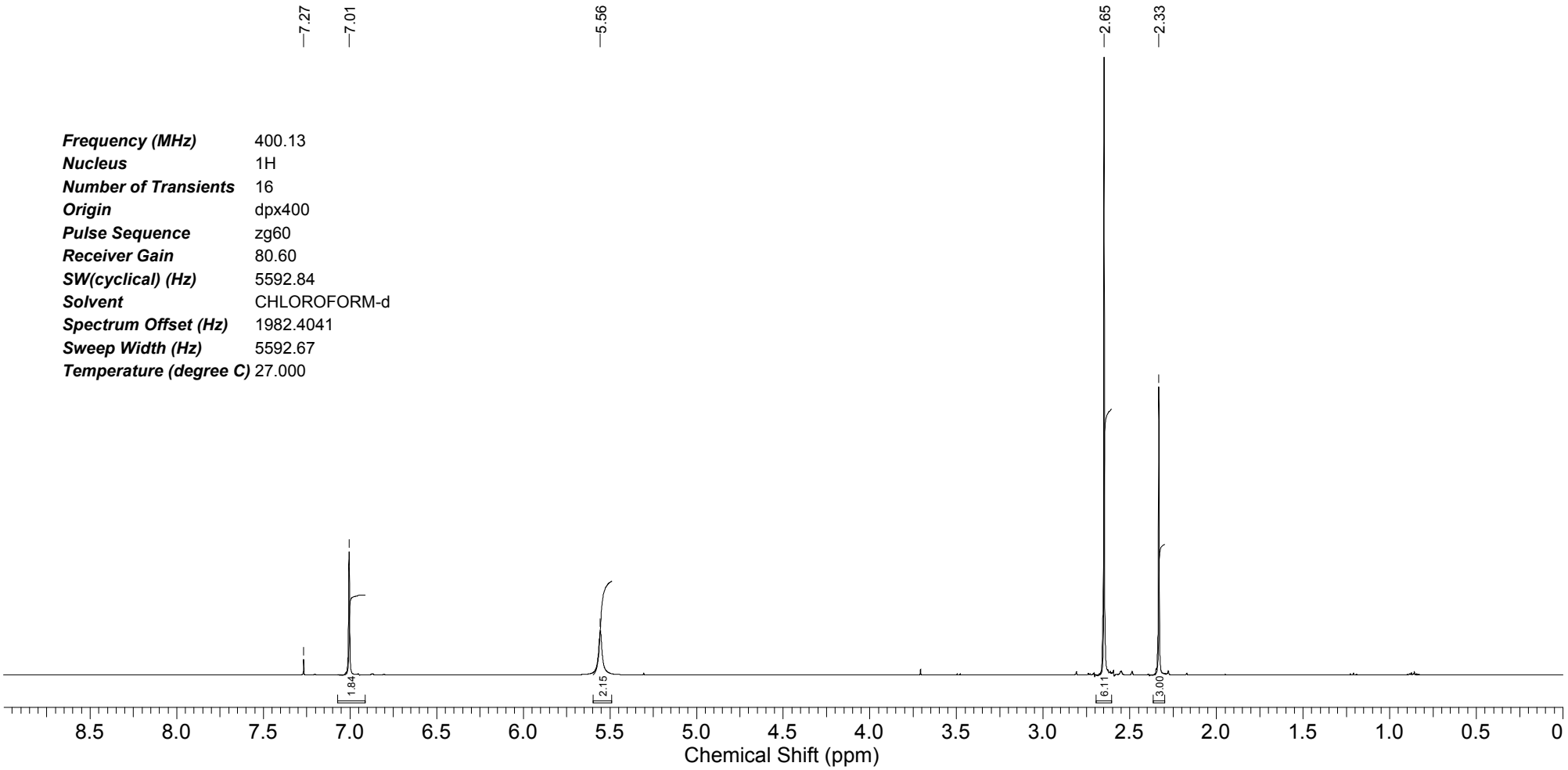


Frequency (MHz)	400.20
Nucleus	1H
Number of Transients	16
Origin	av400
Pulse Sequence	zg60
Receiver Gain	90.50
SW(cyclical) (Hz)	8278.15
Solvent	CHLOROFORM-d
Spectrum Offset (Hz)	2468.5974
Sweep Width (Hz)	8277.89
Temperature (degree C)	27.000

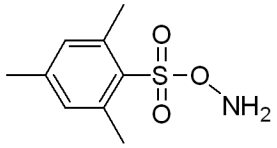


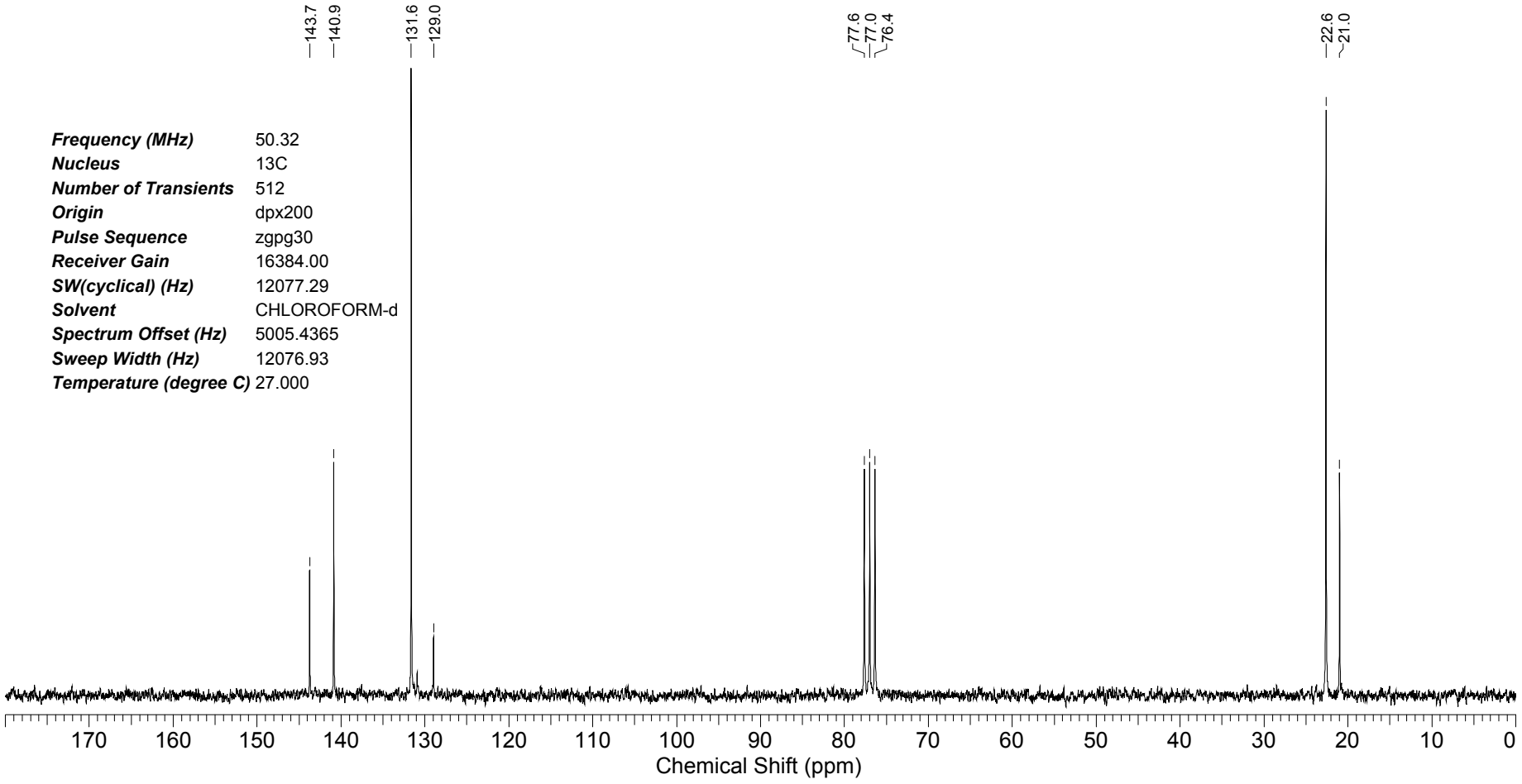


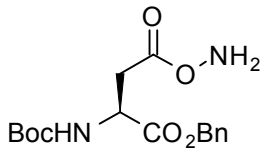


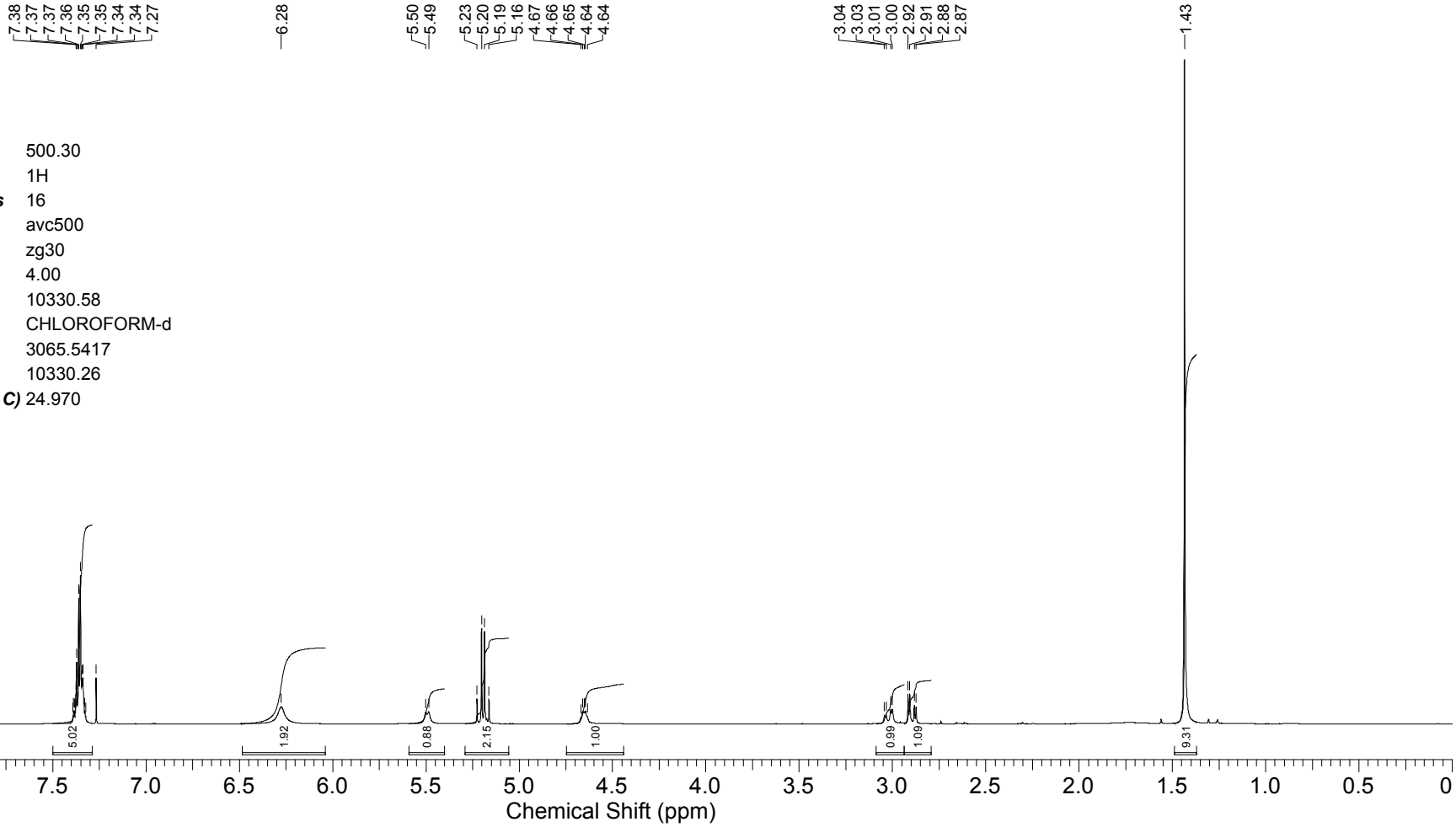


Frequency (MHz)	400.13
Nucleus	1H
Number of Transients	16
Origin	dpx400
Pulse Sequence	zg60
Receiver Gain	80.60
SW(cyclical) (Hz)	5592.84
Solvent	CHLOROFORM-d
Spectrum Offset (Hz)	1982.4041
Sweep Width (Hz)	5592.67
Temperature (degree C)	27.000

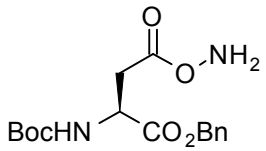






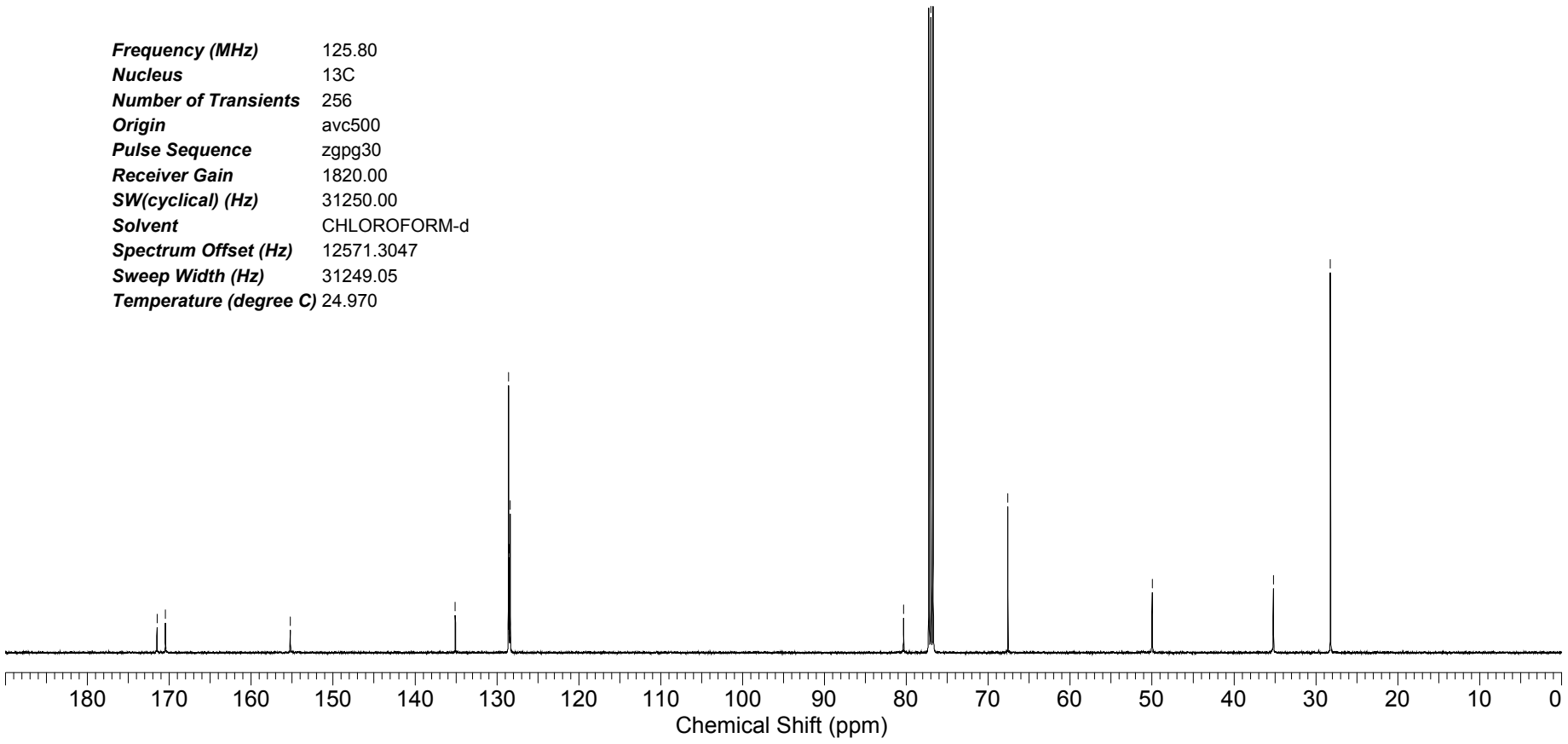


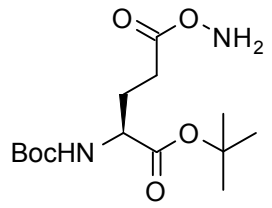
Frequency (MHz)	500.30
Nucleus	1H
Number of Transients	16
Origin	avc500
Pulse Sequence	zg30
Receiver Gain	4.00
SW(cyclical) (Hz)	10330.58
Solvent	CHLOROFORM-d
Spectrum Offset (Hz)	3065.5417
Sweep Width (Hz)	10330.26
Temperature (degree C)	24.970

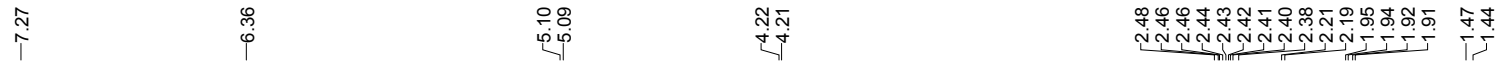


171.5
170.5
155.2
135.1
128.6
128.5
128.4
80.3
77.3
77.0
76.8
67.6
50.0
35.2
28.2

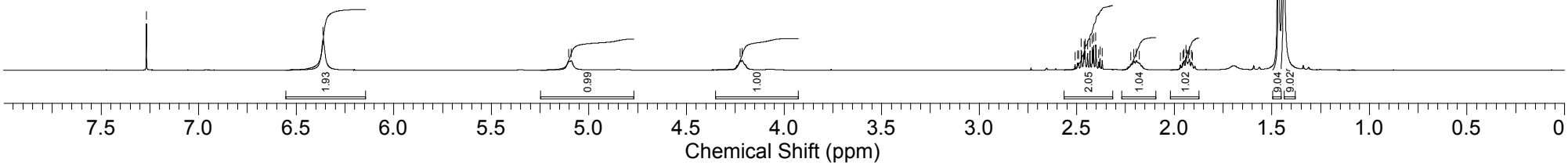
Frequency (MHz)	125.80
Nucleus	13C
Number of Transients	256
Origin	avc500
Pulse Sequence	zgpg30
Receiver Gain	1820.00
SW(cyclical) (Hz)	31250.00
Solvent	CHLOROFORM-d
Spectrum Offset (Hz)	12571.3047
Sweep Width (Hz)	31249.05
Temperature (degree C)	24.970

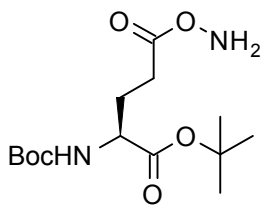




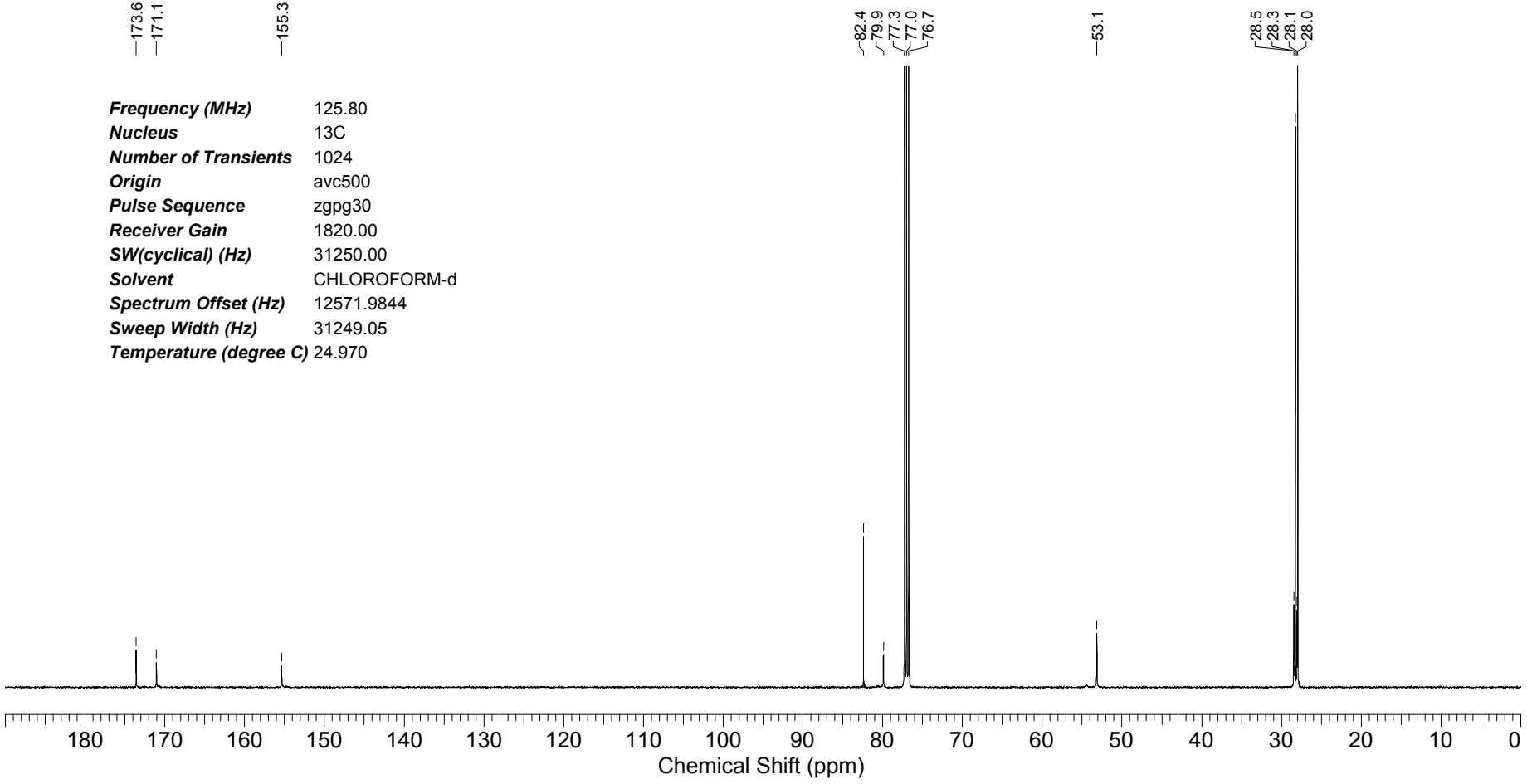


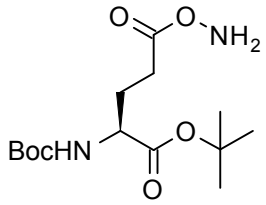
Frequency (MHz)	500.30
Nucleus	1H
Number of Transients	16
Origin	avc500
Pulse Sequence	zg30
Receiver Gain	4.00
SW(cyclical) (Hz)	10330.58
Solvent	CHLOROFORM-d
Spectrum Offset (Hz)	3065.5417
Sweep Width (Hz)	10330.26
Temperature (degree C)	24.970



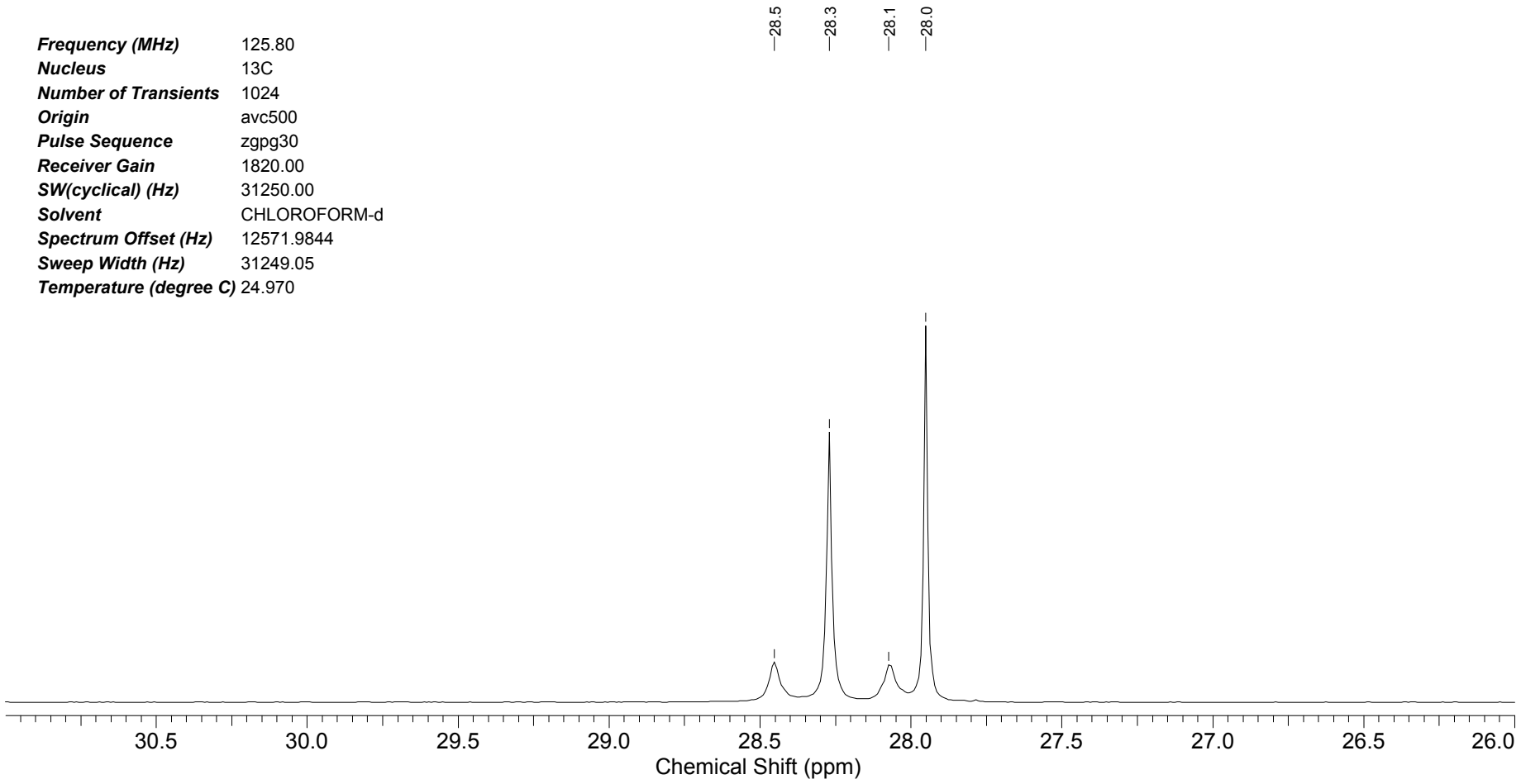


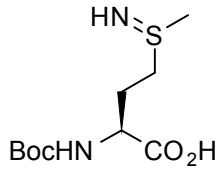
Frequency (MHz)	125.80
Nucleus	13C
Number of Transients	1024
Origin	avc500
Pulse Sequence	zgpg30
Receiver Gain	1820.00
SW(cyclical) (Hz)	31250.00
Solvent	CHLOROFORM-d
Spectrum Offset (Hz)	12571.9844
Sweep Width (Hz)	31249.05
Temperature (degree C)	24.970



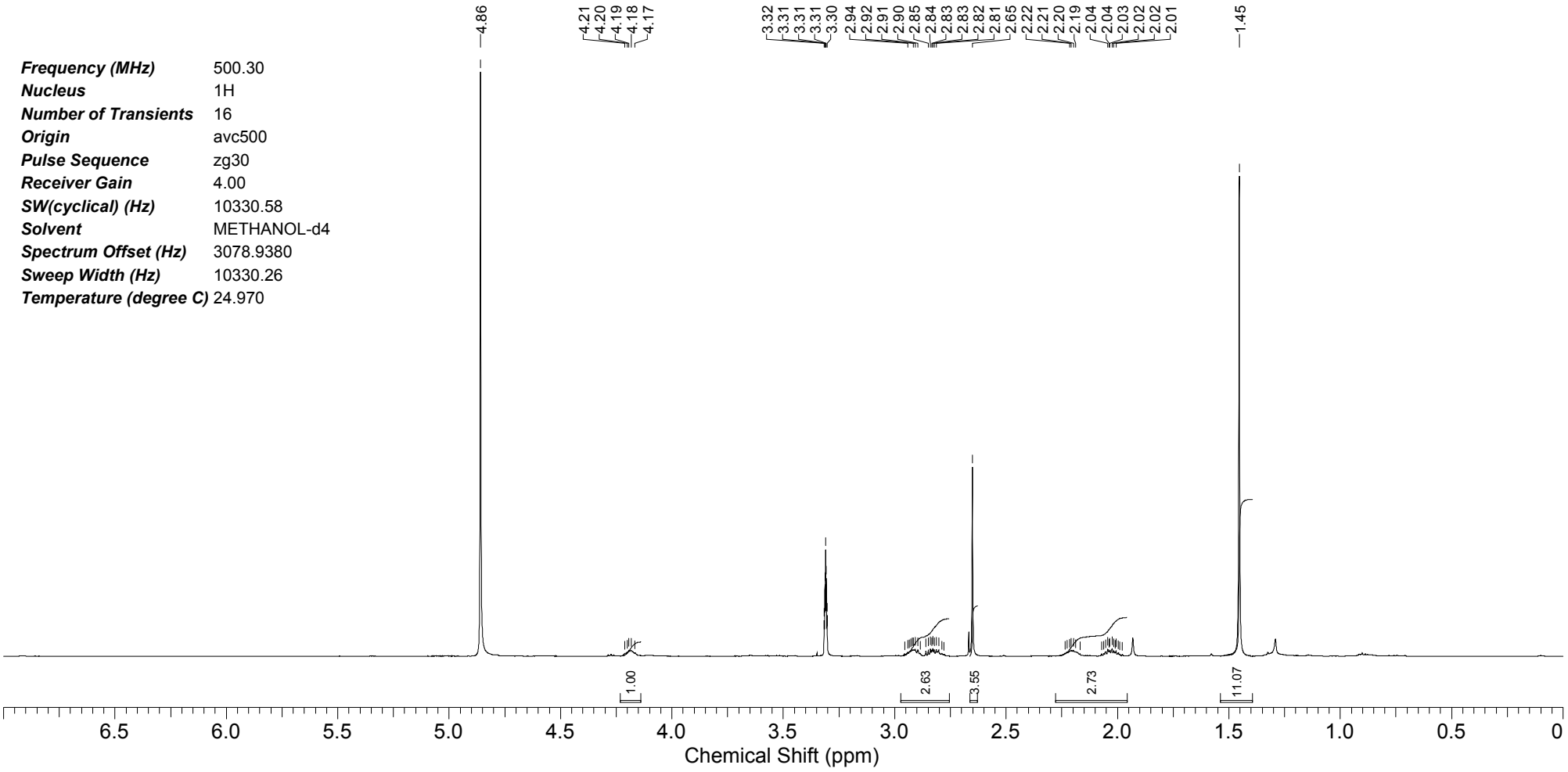


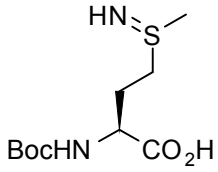
Frequency (MHz)	125.80
Nucleus	13C
Number of Transients	1024
Origin	avc500
Pulse Sequence	zgpg30
Receiver Gain	1820.00
SW(cyclical) (Hz)	31250.00
Solvent	CHLOROFORM-d
Spectrum Offset (Hz)	12571.9844
Sweep Width (Hz)	31249.05
Temperature (degree C)	24.970



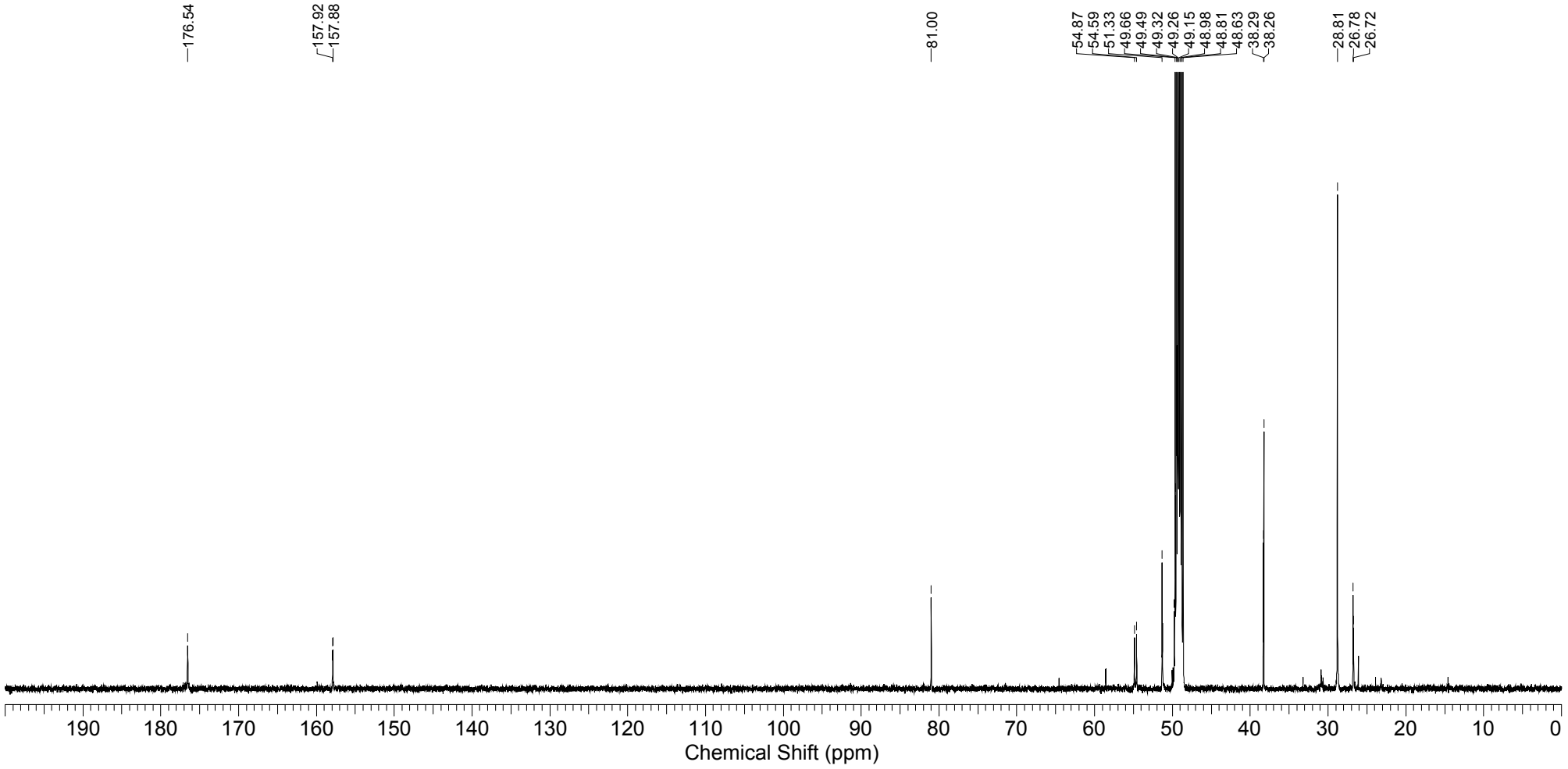


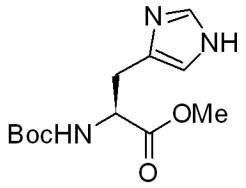
Frequency (MHz)	500.30
Nucleus	1H
Number of Transients	16
Origin	avc500
Pulse Sequence	zg30
Receiver Gain	4.00
SW(cyclical) (Hz)	10330.58
Solvent	METHANOL-d4
Spectrum Offset (Hz)	3078.9380
Sweep Width (Hz)	10330.26
Temperature (degree C)	24.970

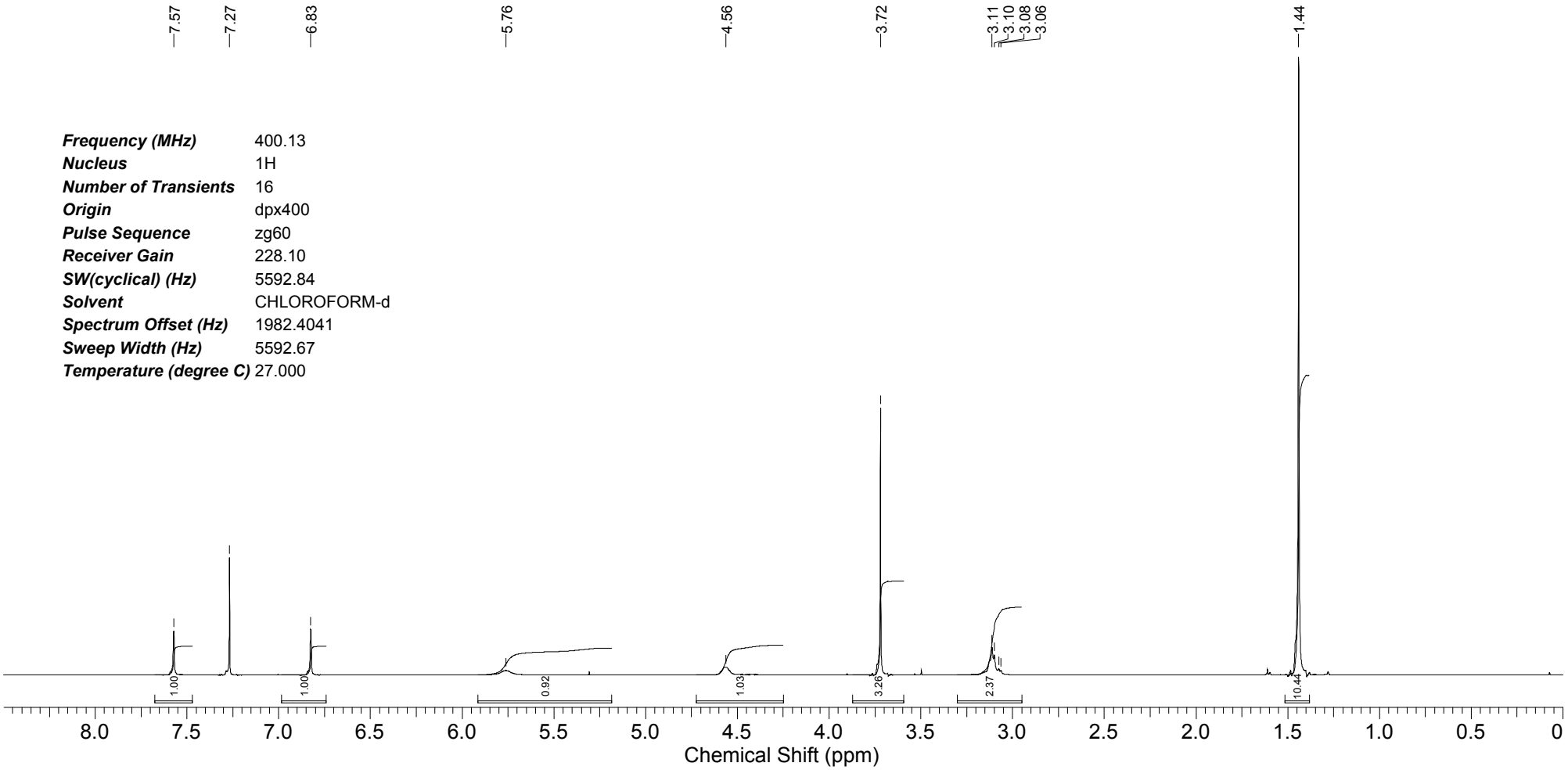




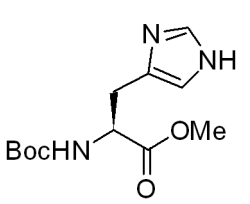
Frequency (MHz)	125.80
Nucleus	13C
Number of Transients	1024
Origin	avc500
Pulse Sequence	zgpg30
Receiver Gain	1820.00
SW(cyclical) (Hz)	31250.00
Solvent	METHANOL-d4
Spectrum Offset (Hz)	12774.4082
Sweep Width (Hz)	31249.05
Temperature (degree C)	24.970

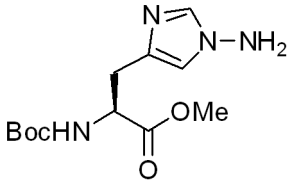


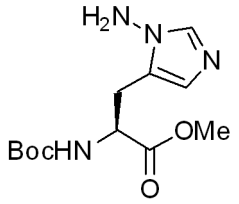




Frequency (MHz)	400.13
Nucleus	1H
Number of Transients	16
Origin	dpx400
Pulse Sequence	zg60
Receiver Gain	228.10
SW(cyclical) (Hz)	5592.84
Solvent	CHLOROFORM-d
Spectrum Offset (Hz)	1982.4041
Sweep Width (Hz)	5592.67
Temperature (degree C)	27.000

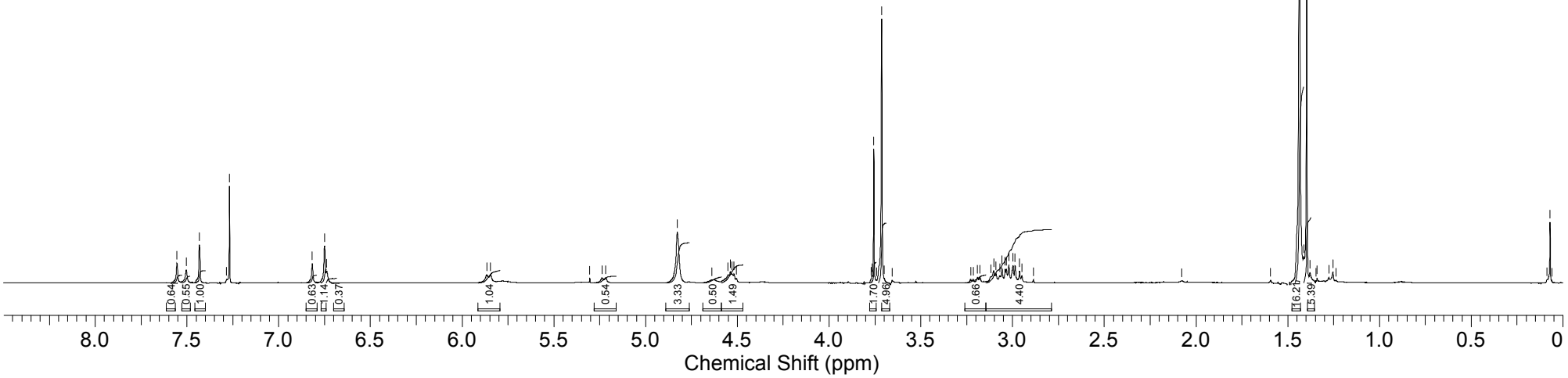


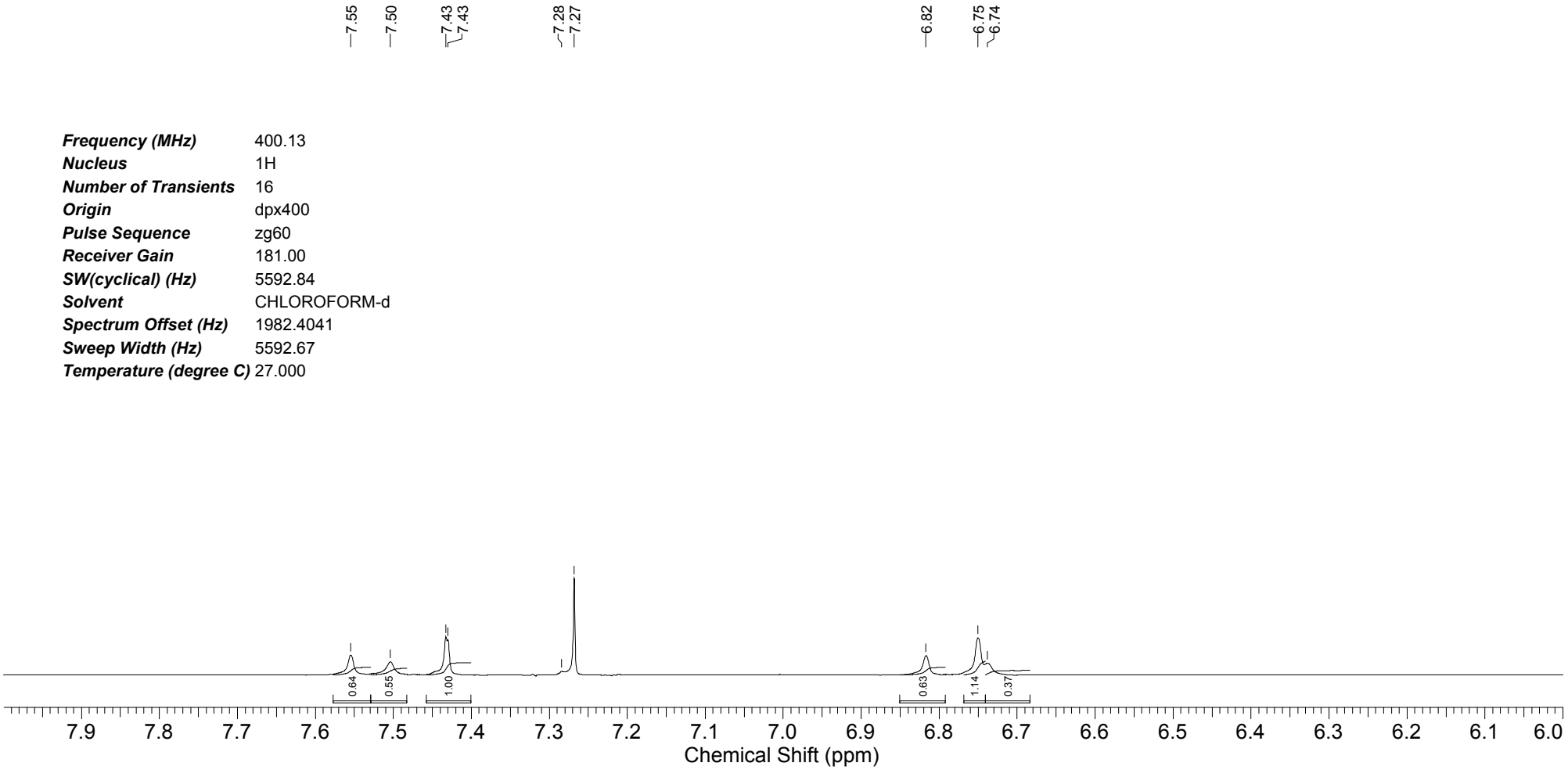




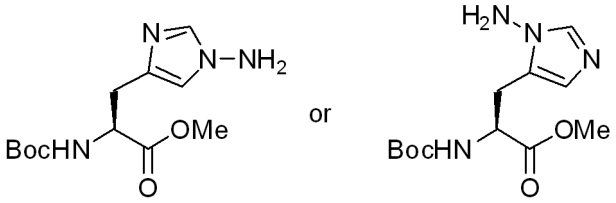
7.55 7.50 7.43 7.28 7.27 6.82 6.75 6.74 5.87 5.85 5.30 5.24 5.22 4.83 4.64 4.55 4.54 4.53 4.52 4.51 3.77 3.77 3.76 3.71 3.70 3.22 3.12 3.10 3.07 3.06 3.04 3.03 3.02 3.00 2.99 2.96 2.08 1.59 1.44 1.41 1.40 1.38 1.35 1.34 1.28 1.25 1.24 0.09 0.07

Frequency (MHz)	400.13
Nucleus	1H
Number of Transients	16
Origin	dpx400
Pulse Sequence	zg60
Receiver Gain	181.00
SW(cyclical) (Hz)	5592.84
Solvent	CHLOROFORM-d
Spectrum Offset (Hz)	1982.4041
Sweep Width (Hz)	5592.67
Temperature (degree C)	27.000



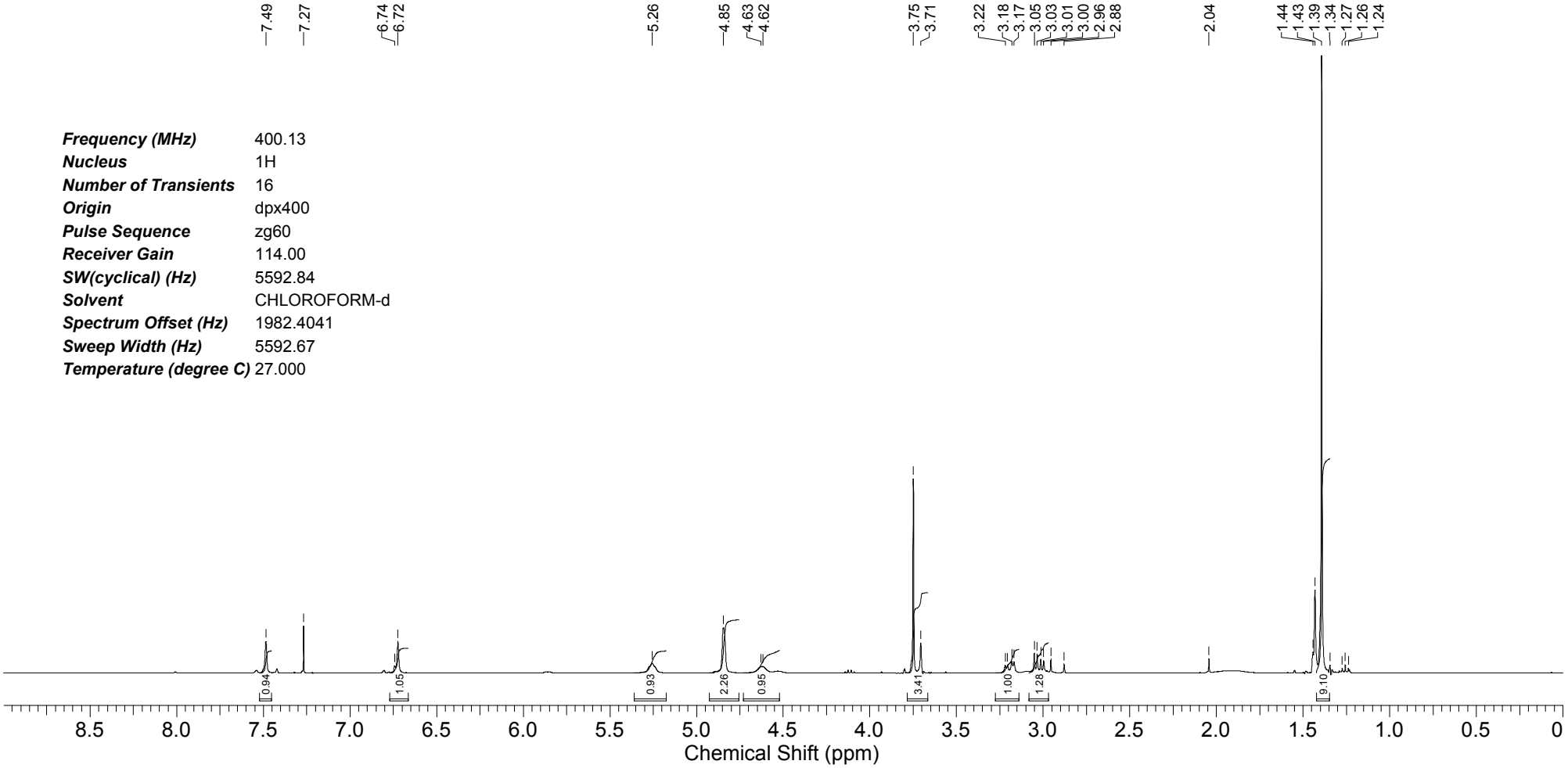


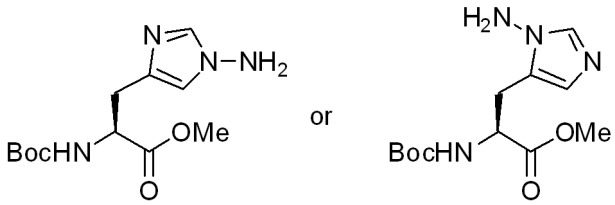
Frequency (MHz)	400.13
Nucleus	1H
Number of Transients	16
Origin	dpx400
Pulse Sequence	zg60
Receiver Gain	181.00
SW(cyclical) (Hz)	5592.84
Solvent	CHLOROFORM-d
Spectrum Offset (Hz)	1982.4041
Sweep Width (Hz)	5592.67
Temperature (degree C)	27.000



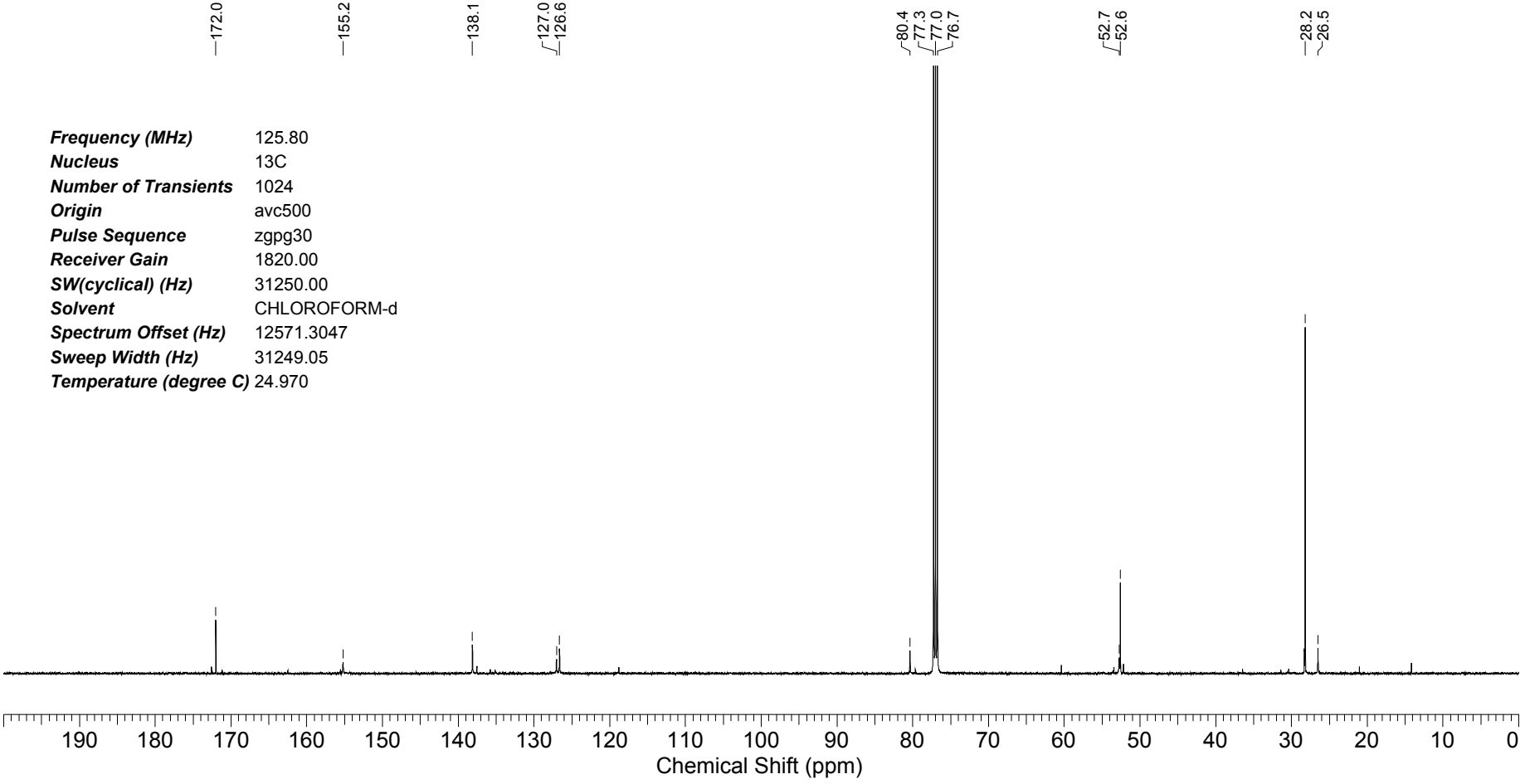
or

Frequency (MHz)	400.13
Nucleus	1H
Number of Transients	16
Origin	dpx400
Pulse Sequence	zg60
Receiver Gain	114.00
SW(cyclical) (Hz)	5592.84
Solvent	CHLOROFORM-d
Spectrum Offset (Hz)	1982.4041
Sweep Width (Hz)	5592.67
Temperature (degree C)	27.000

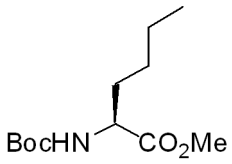


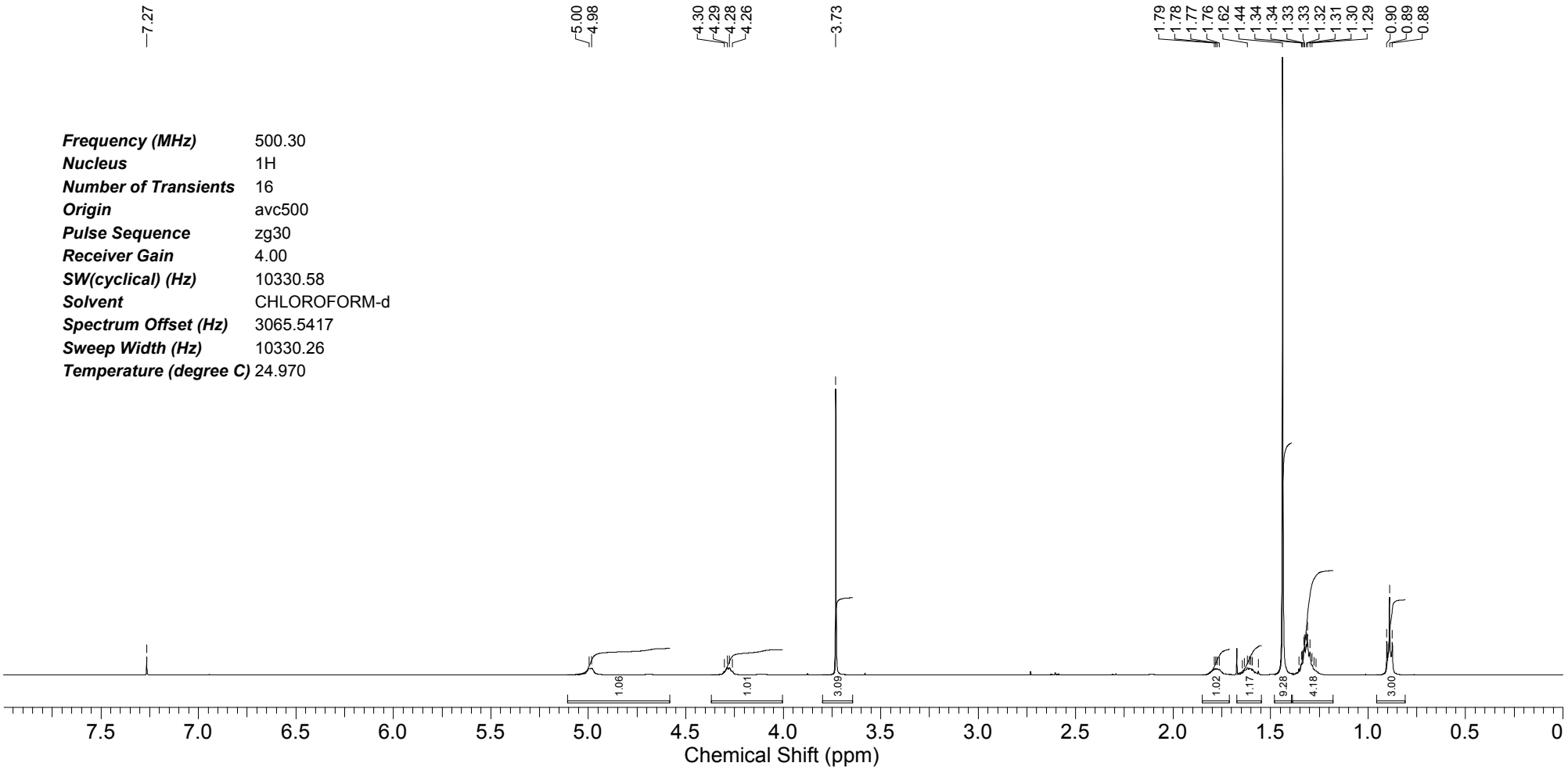


or

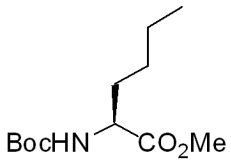


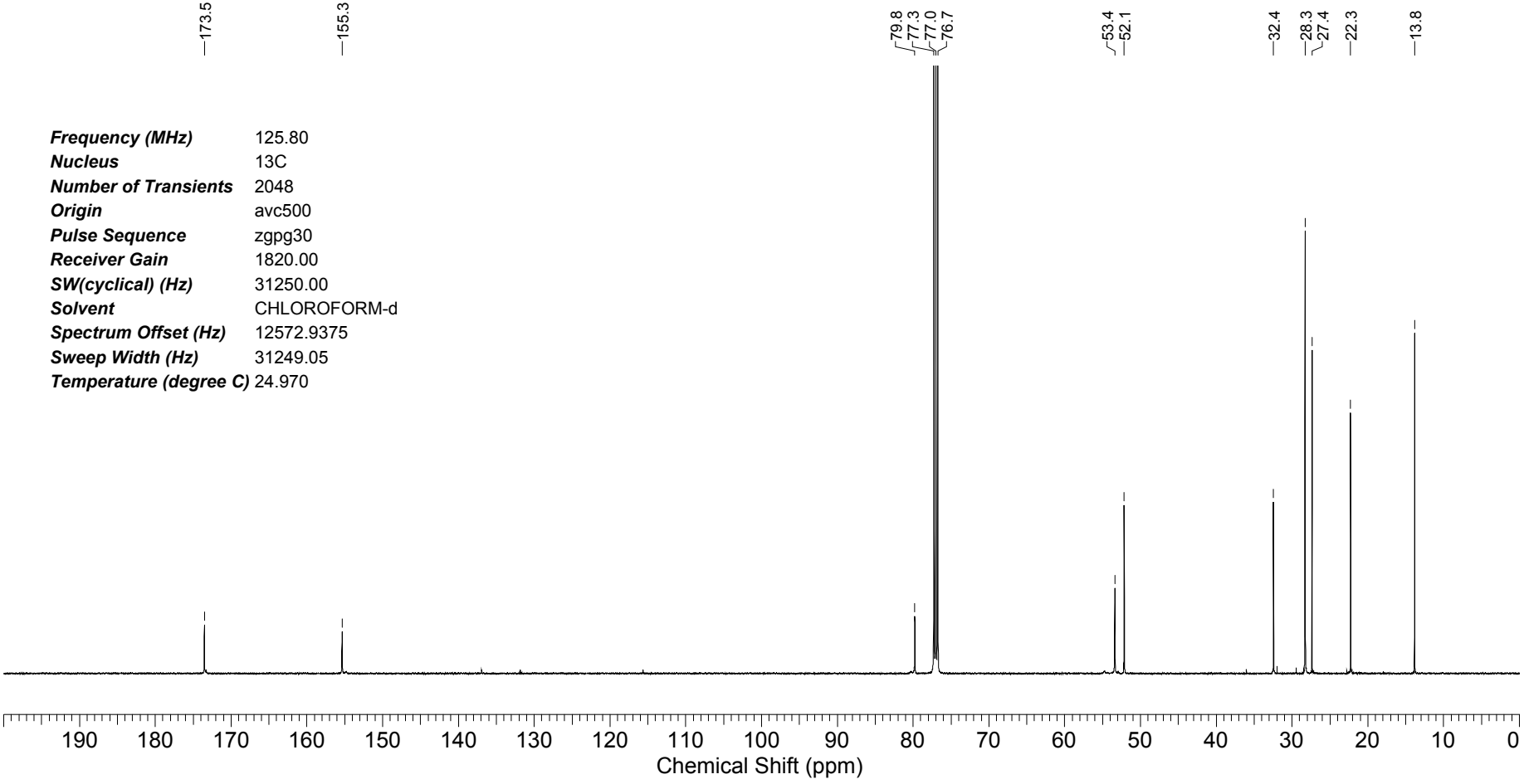
Frequency (MHz)	125.80
Nucleus	13C
Number of Transients	1024
Origin	avc500
Pulse Sequence	zgpg30
Receiver Gain	1820.00
SW(cyclical) (Hz)	31250.00
Solvent	CHLOROFORM-d
Spectrum Offset (Hz)	12571.3047
Sweep Width (Hz)	31249.05
Temperature (degree C)	24.970



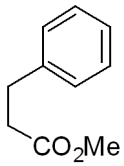


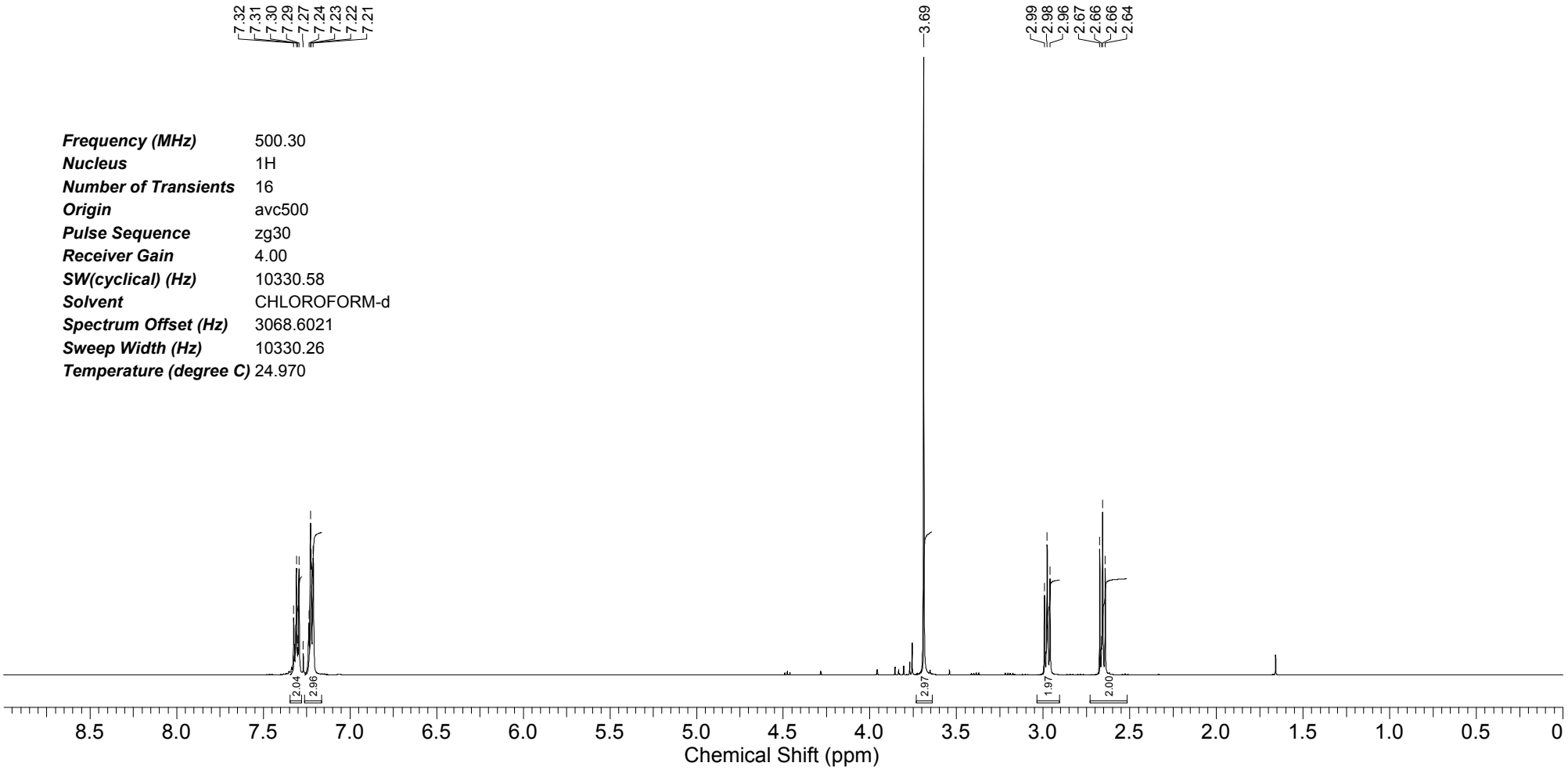
Frequency (MHz)	500.30
Nucleus	1H
Number of Transients	16
Origin	avc500
Pulse Sequence	zg30
Receiver Gain	4.00
SW(cyclical) (Hz)	10330.58
Solvent	CHLOROFORM-d
Spectrum Offset (Hz)	3065.5417
Sweep Width (Hz)	10330.26
Temperature (degree C)	24.970



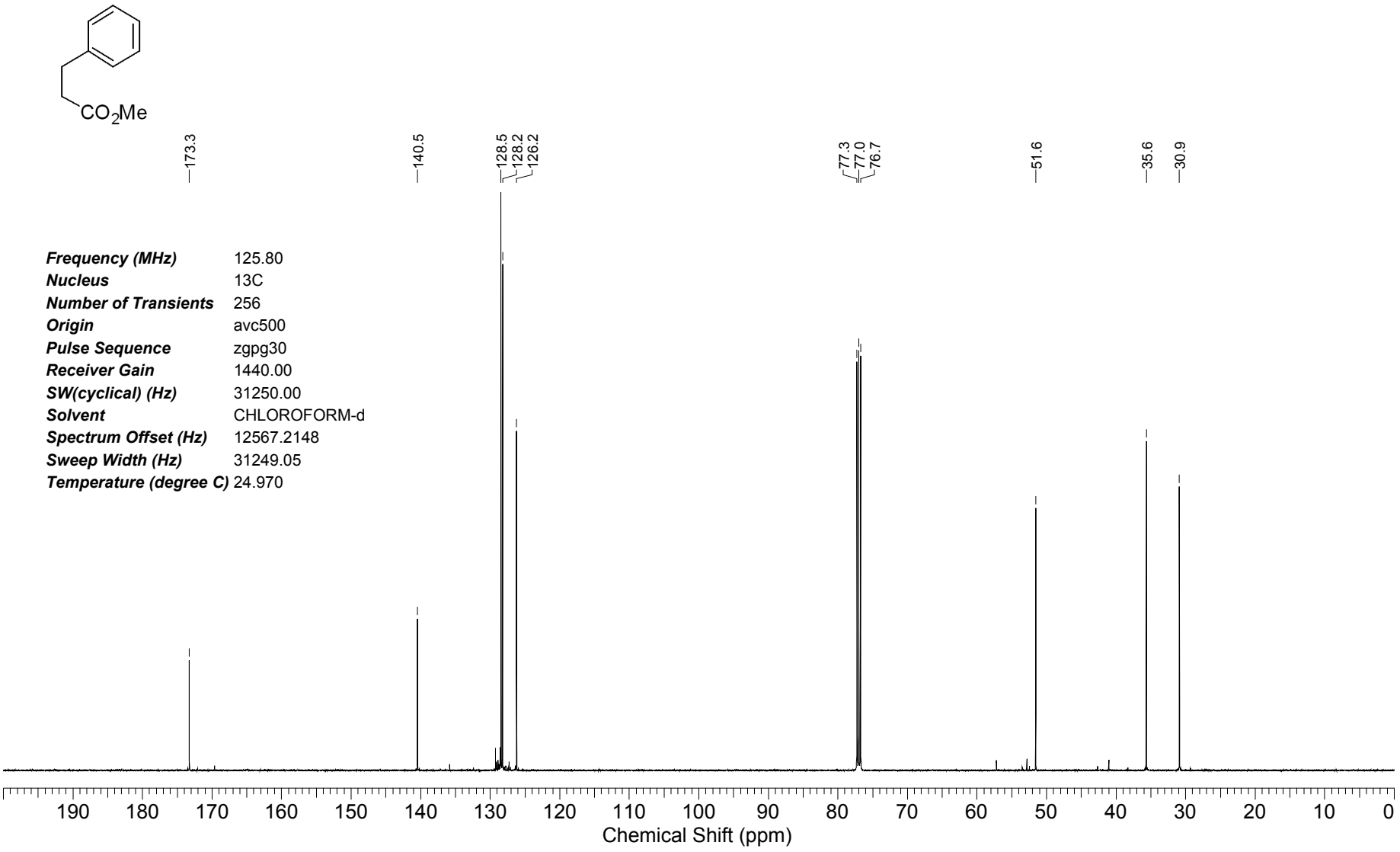


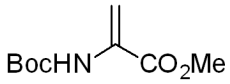
Frequency (MHz)	125.80
Nucleus	13C
Number of Transients	2048
Origin	avc500
Pulse Sequence	zgpg30
Receiver Gain	1820.00
SW(cyclical) (Hz)	31250.00
Solvent	CHLOROFORM-d
Spectrum Offset (Hz)	12572.9375
Sweep Width (Hz)	31249.05
Temperature (degree C)	24.970

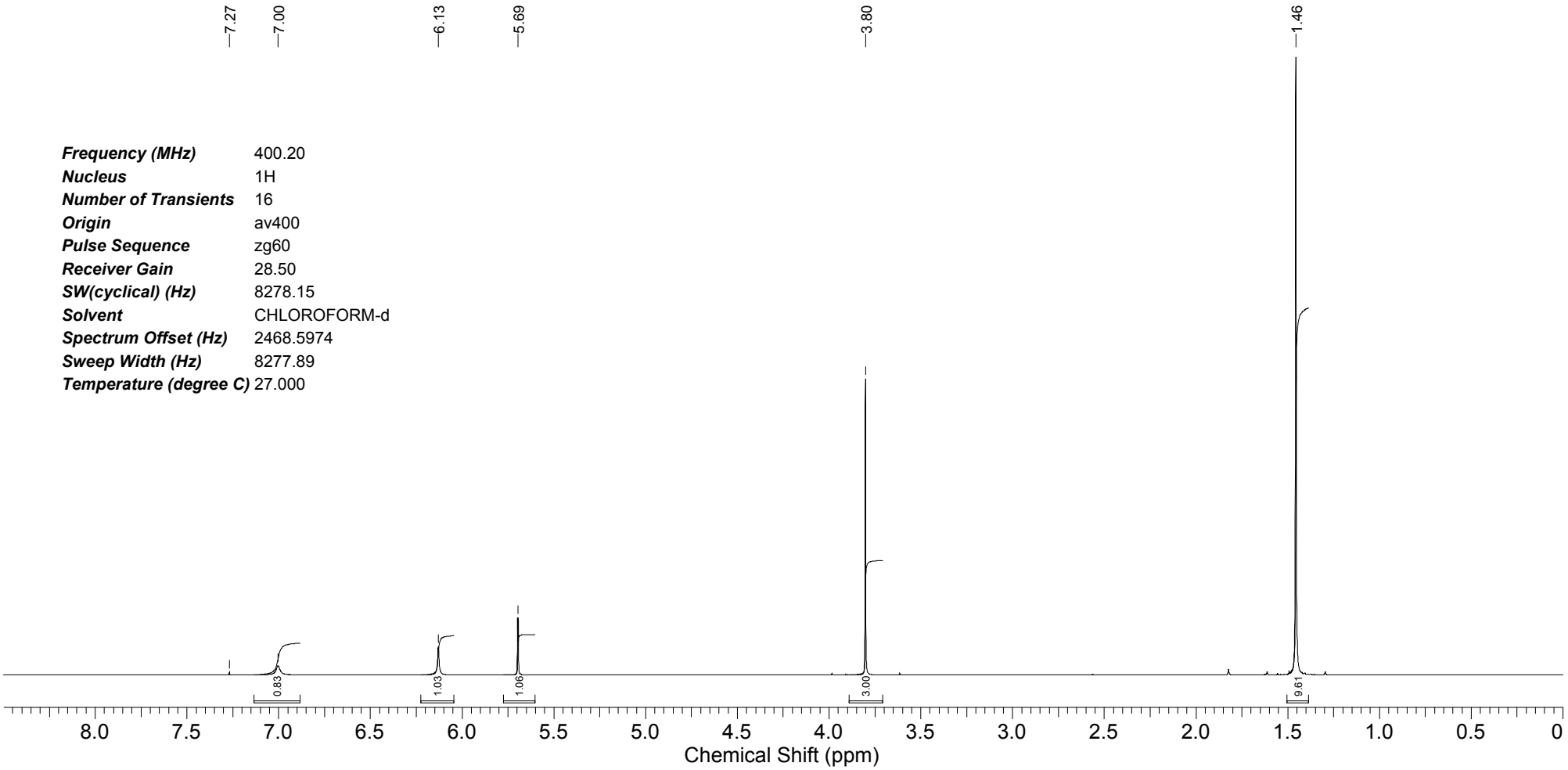


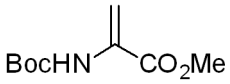


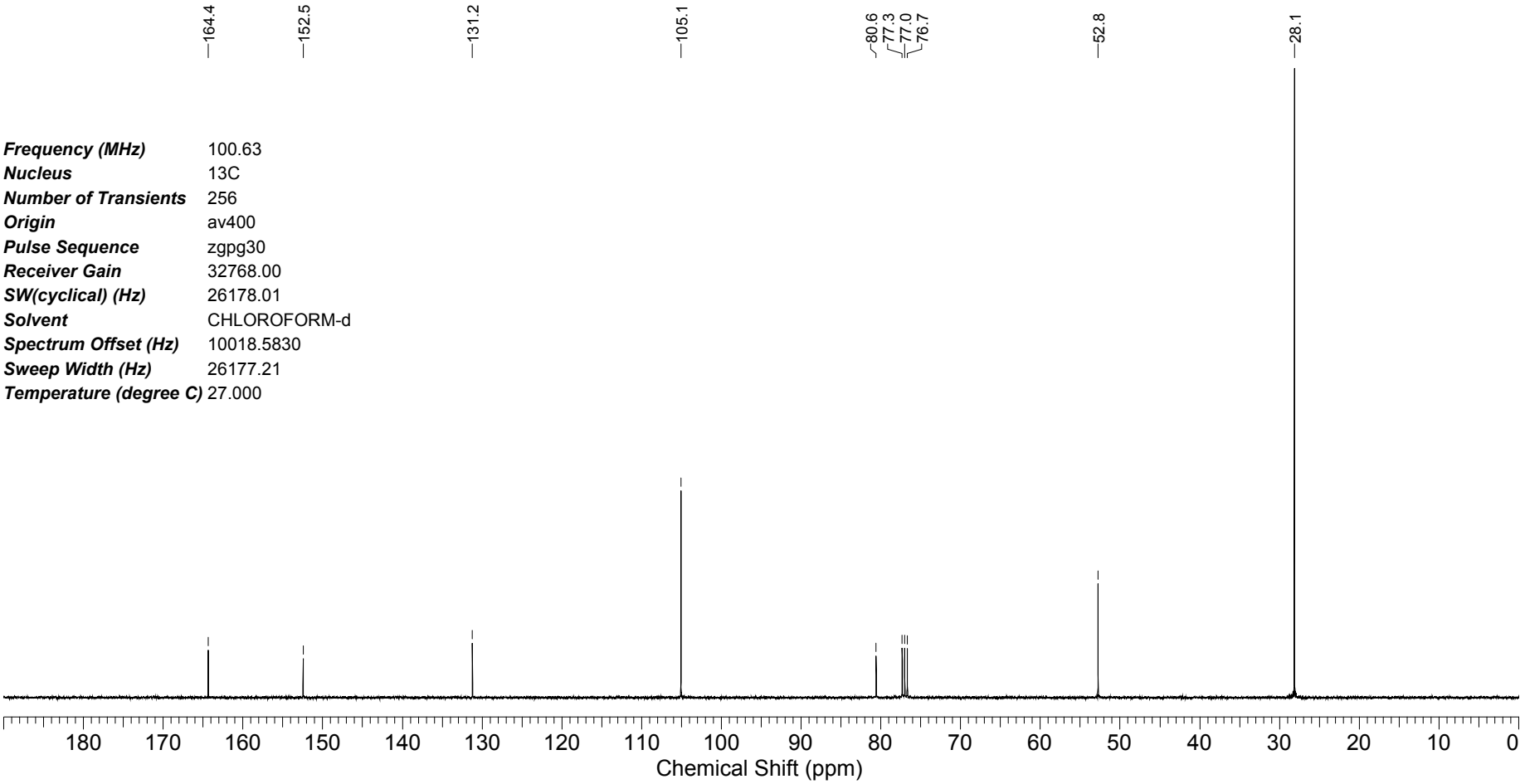
Frequency (MHz)	500.30
Nucleus	1H
Number of Transients	16
Origin	avc500
Pulse Sequence	zg30
Receiver Gain	4.00
SW(cyclical) (Hz)	10330.58
Solvent	CHLOROFORM-d
Spectrum Offset (Hz)	3068.6021
Sweep Width (Hz)	10330.26
Temperature (degree C)	24.970



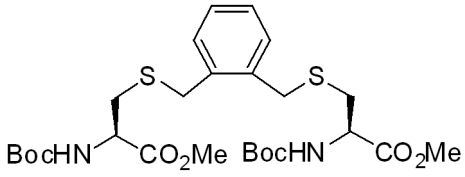




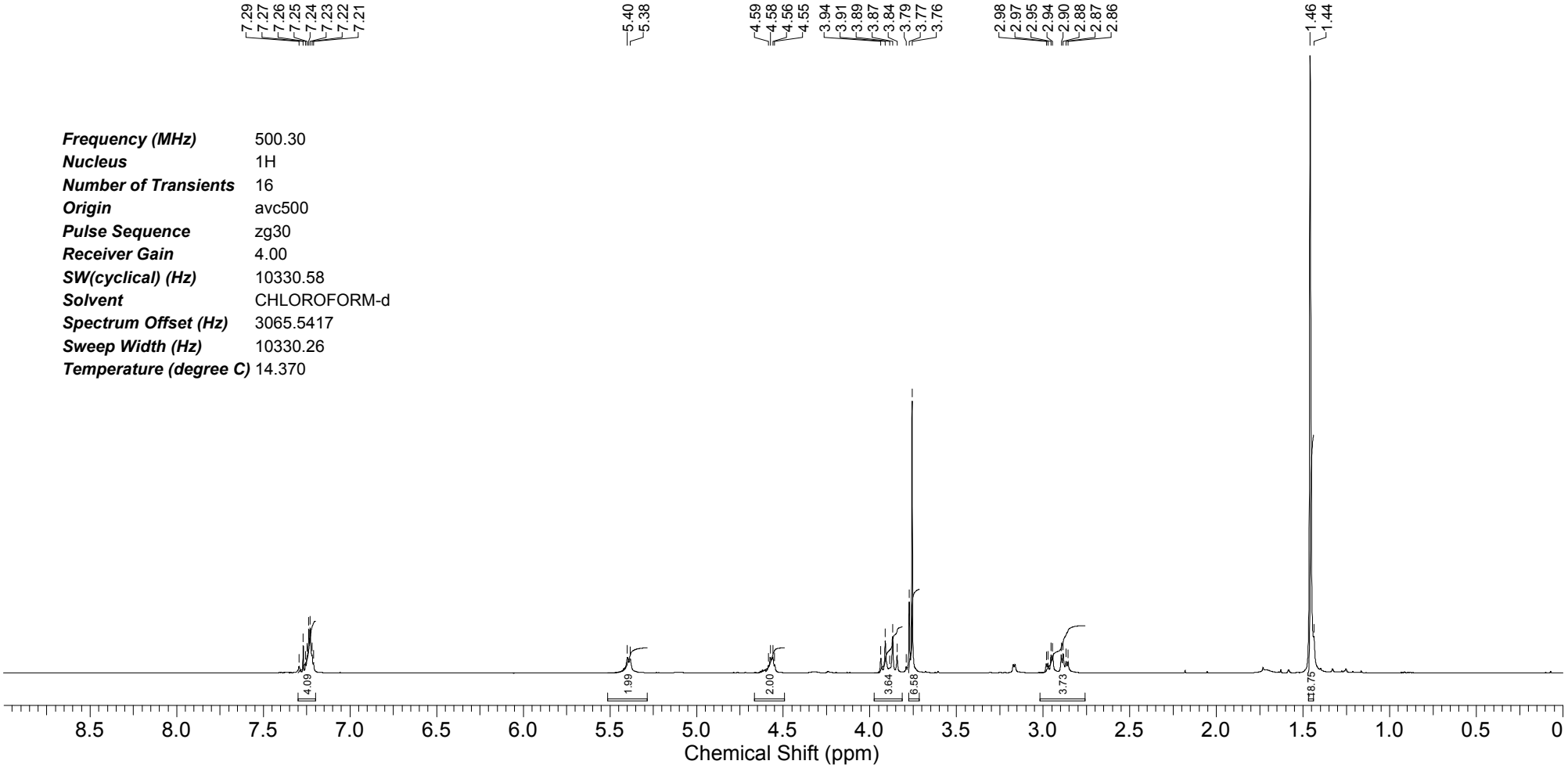


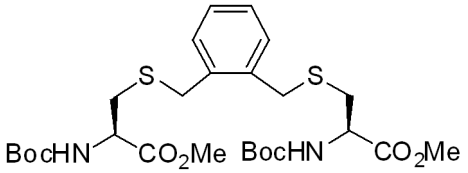


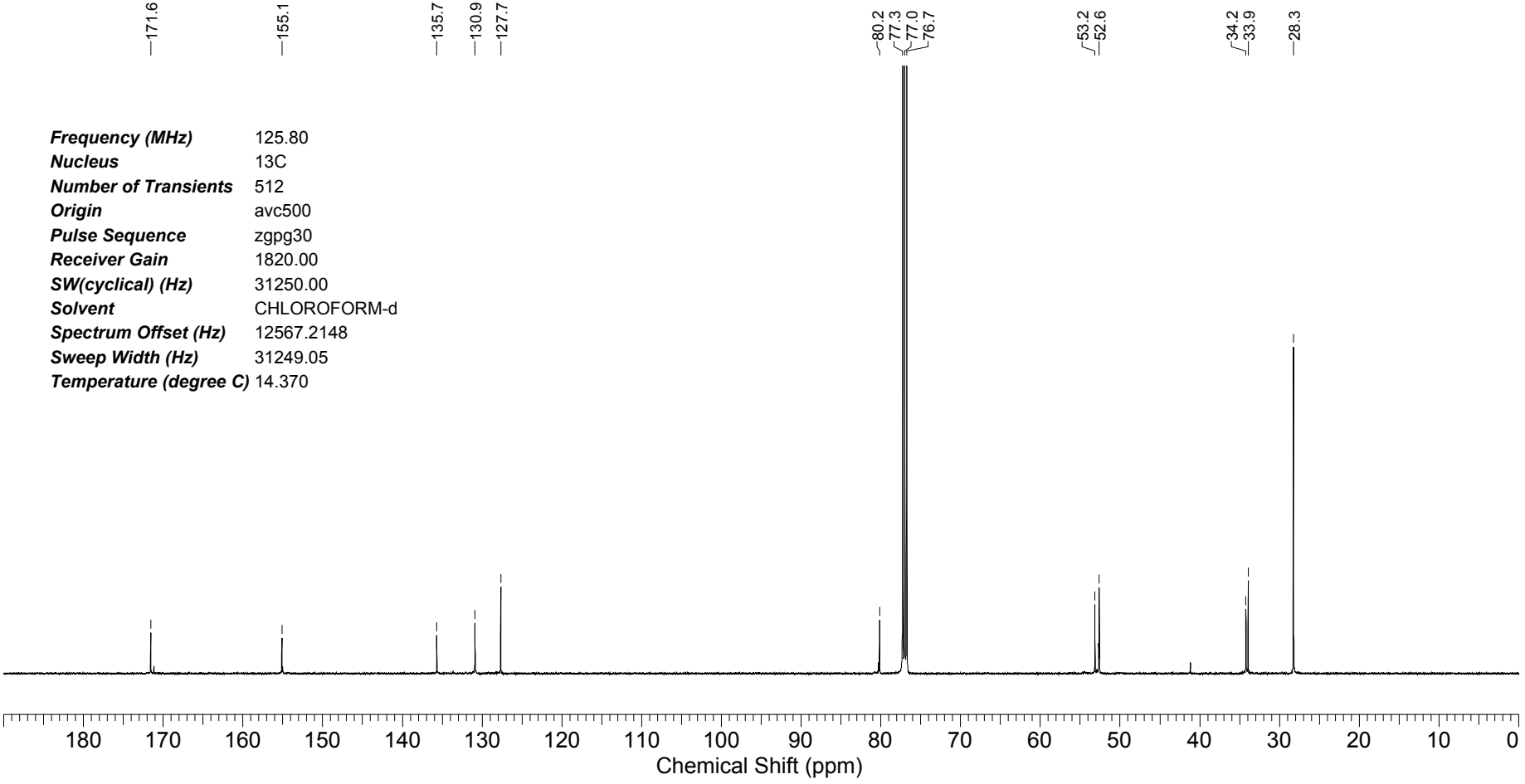
Frequency (MHz)	100.63
Nucleus	13C
Number of Transients	256
Origin	av400
Pulse Sequence	zgpg30
Receiver Gain	32768.00
SW(cyclical) (Hz)	26178.01
Solvent	CHLOROFORM-d
Spectrum Offset (Hz)	10018.5830
Sweep Width (Hz)	26177.21
Temperature (degree C)	27.000



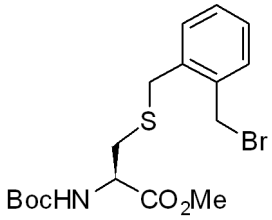
Frequency (MHz)	500.30
Nucleus	1H
Number of Transients	16
Origin	avc500
Pulse Sequence	zg30
Receiver Gain	4.00
SW(cyclical) (Hz)	10330.58
Solvent	CHLOROFORM-d
Spectrum Offset (Hz)	3065.5417
Sweep Width (Hz)	10330.26
Temperature (degree C)	14.370



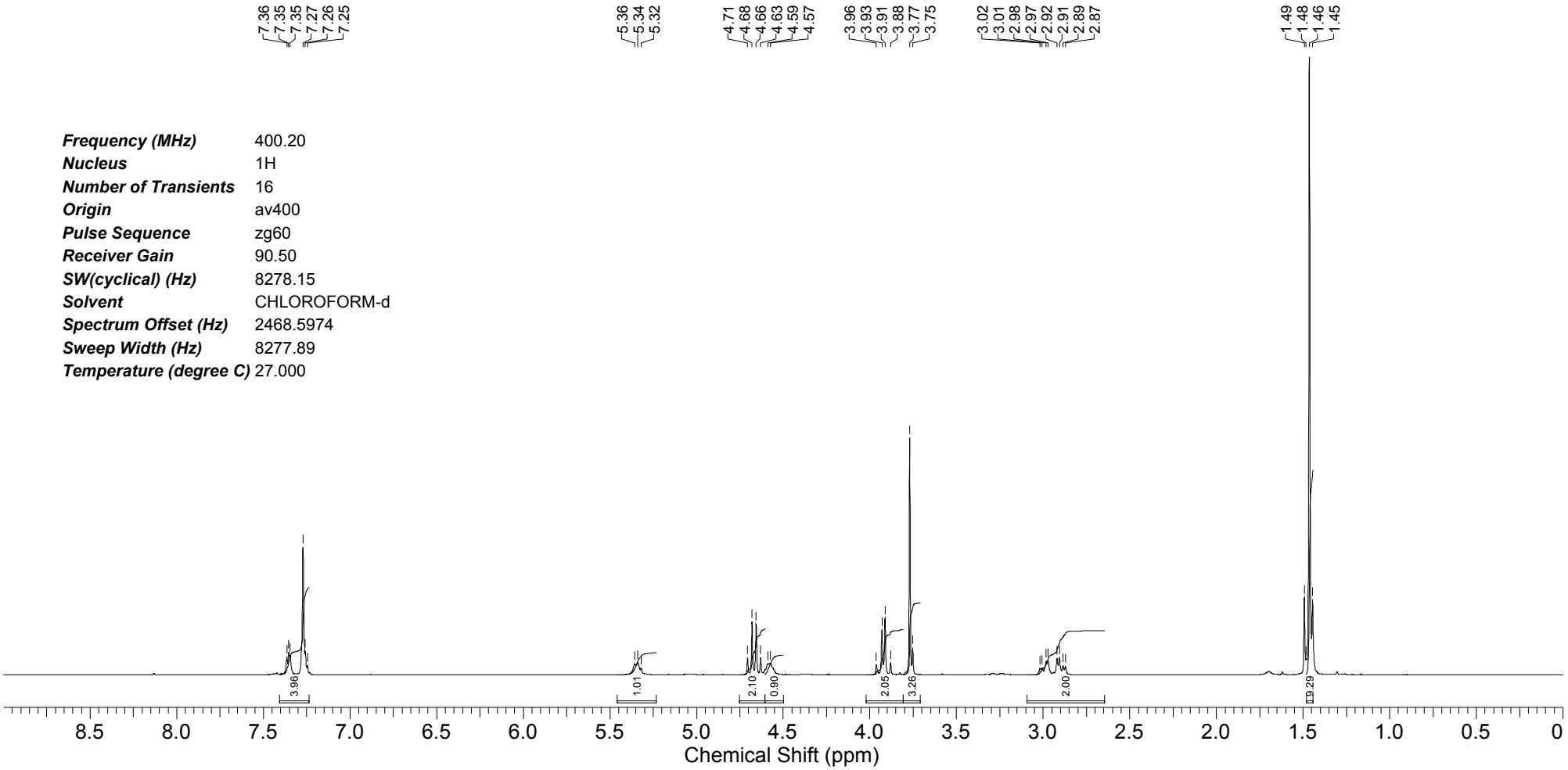


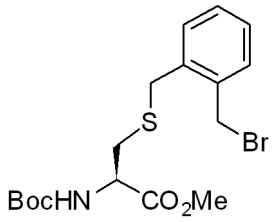


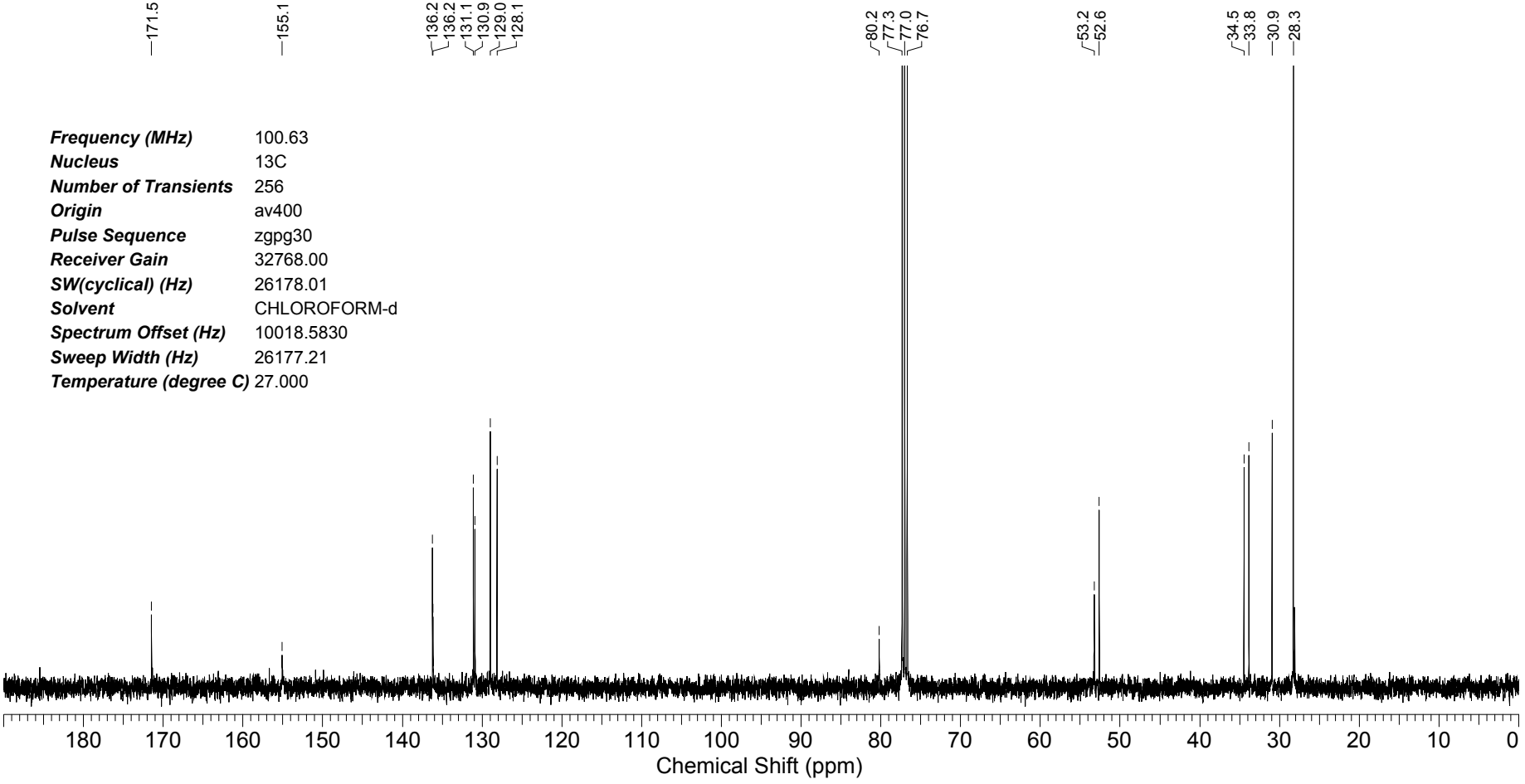
Frequency (MHz)	125.80
Nucleus	13C
Number of Transients	512
Origin	avc500
Pulse Sequence	zgpg30
Receiver Gain	1820.00
SW(cyclical) (Hz)	31250.00
Solvent	CHLOROFORM-d
Spectrum Offset (Hz)	12567.2148
Sweep Width (Hz)	31249.05
Temperature (degree C)	14.370

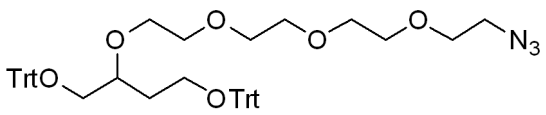


Frequency (MHz)	400.20
Nucleus	1H
Number of Transients	16
Origin	av400
Pulse Sequence	zg60
Receiver Gain	90.50
SW(cyclical) (Hz)	8278.15
Solvent	CHLOROFORM-d
Spectrum Offset (Hz)	2468.5974
Sweep Width (Hz)	8277.89
Temperature (degree C)	27.000

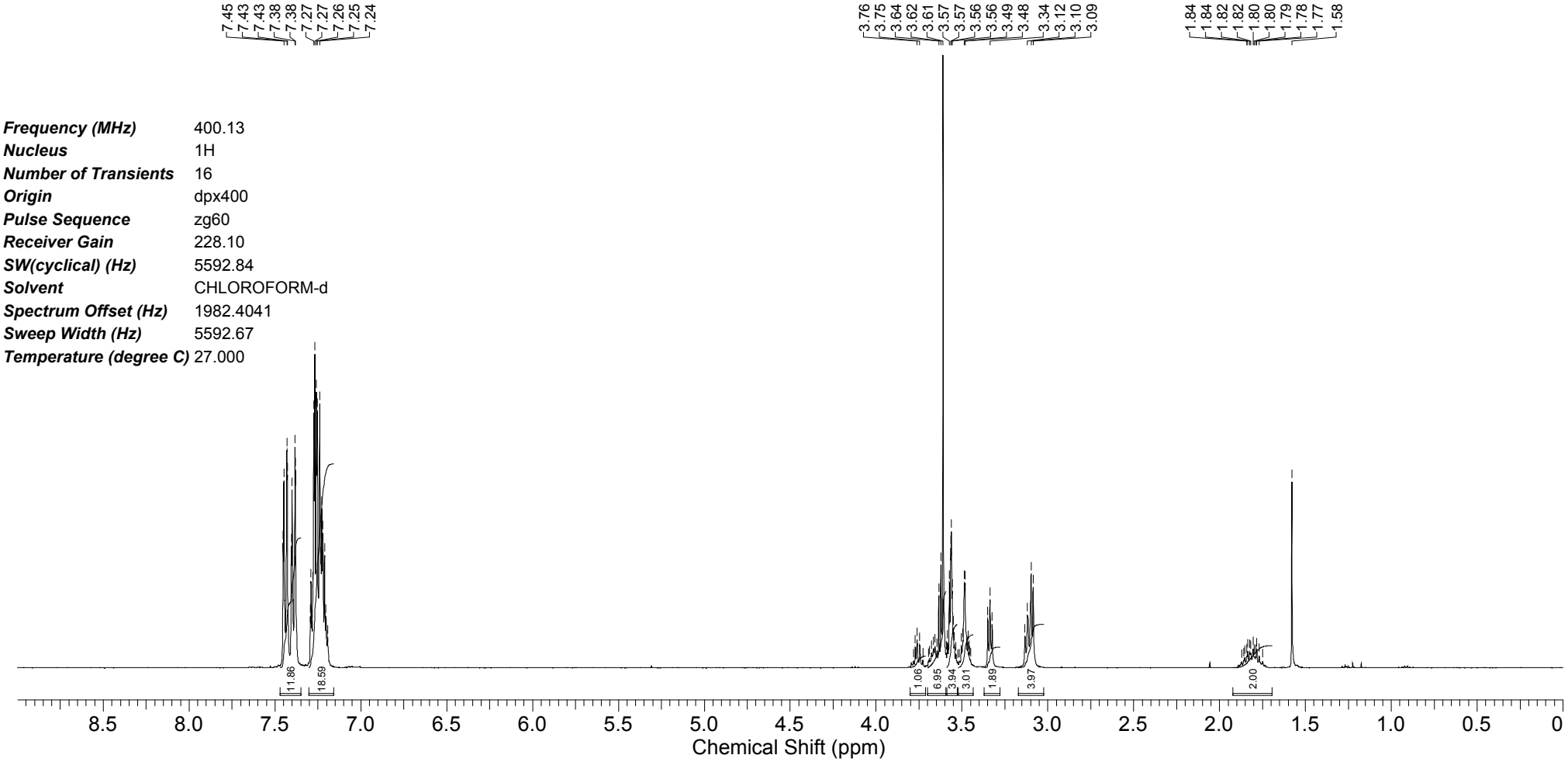


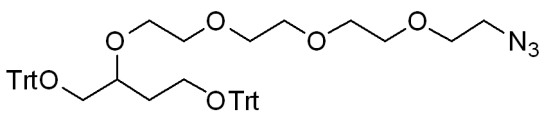


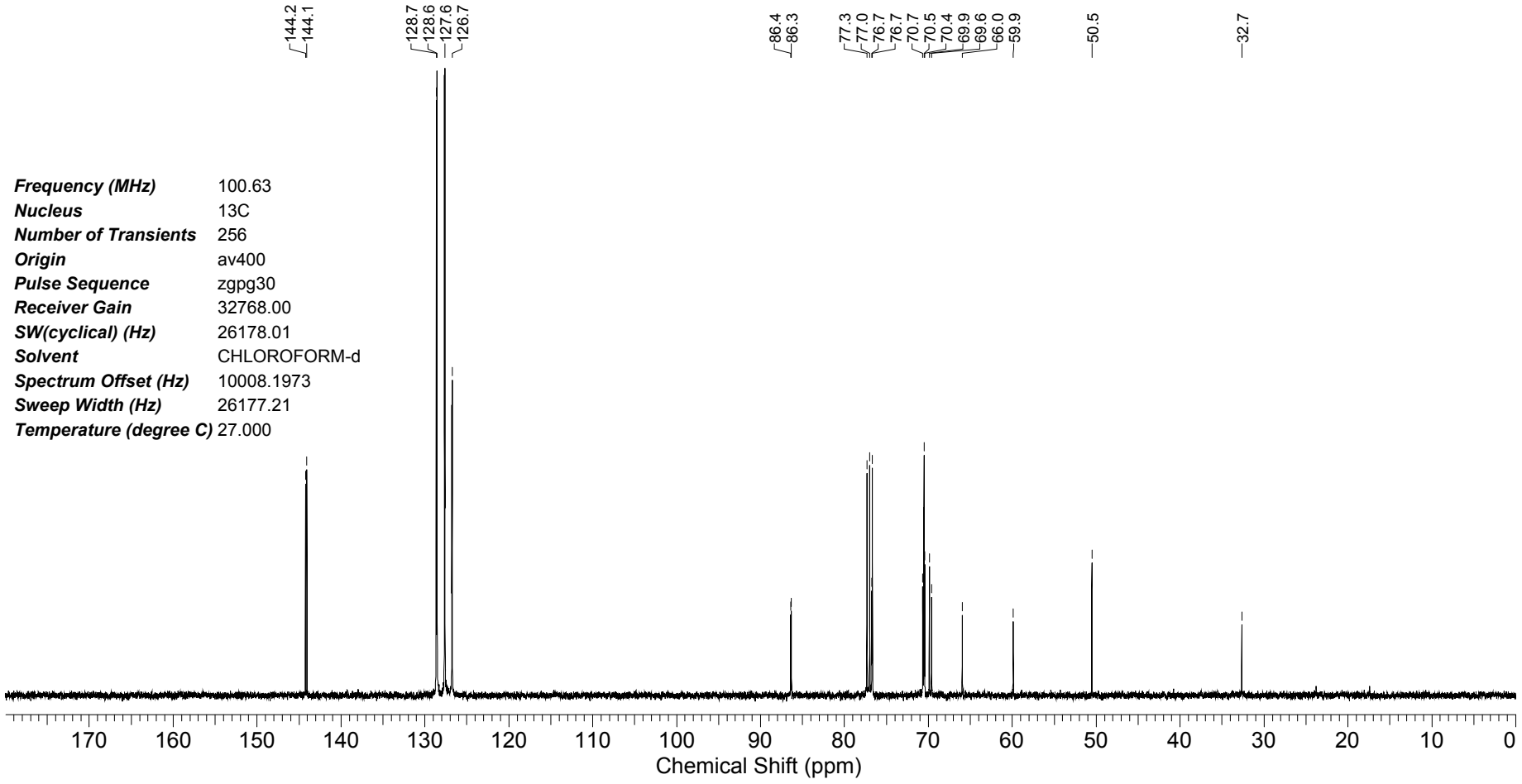


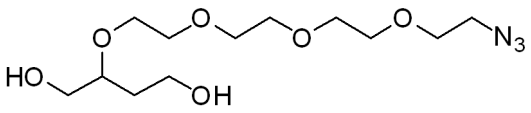


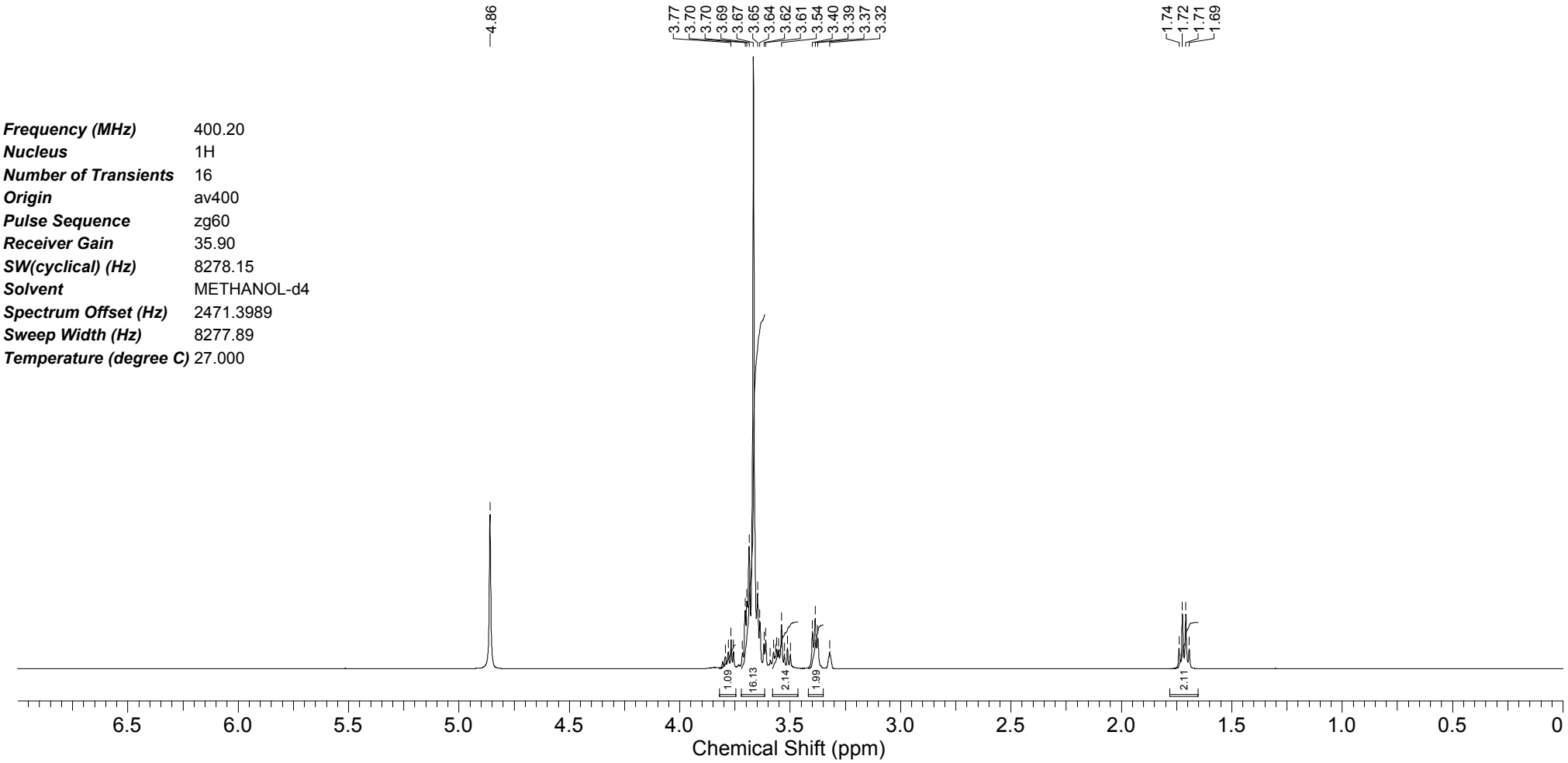
Frequency (MHz)	400.13
Nucleus	1H
Number of Transients	16
Origin	dpx400
Pulse Sequence	zg60
Receiver Gain	228.10
SW(cyclical) (Hz)	5592.84
Solvent	CHLOROFORM-d
Spectrum Offset (Hz)	1982.4041
Sweep Width (Hz)	5592.67
Temperature (degree C)	27.000



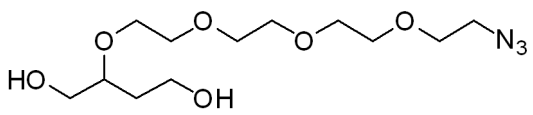


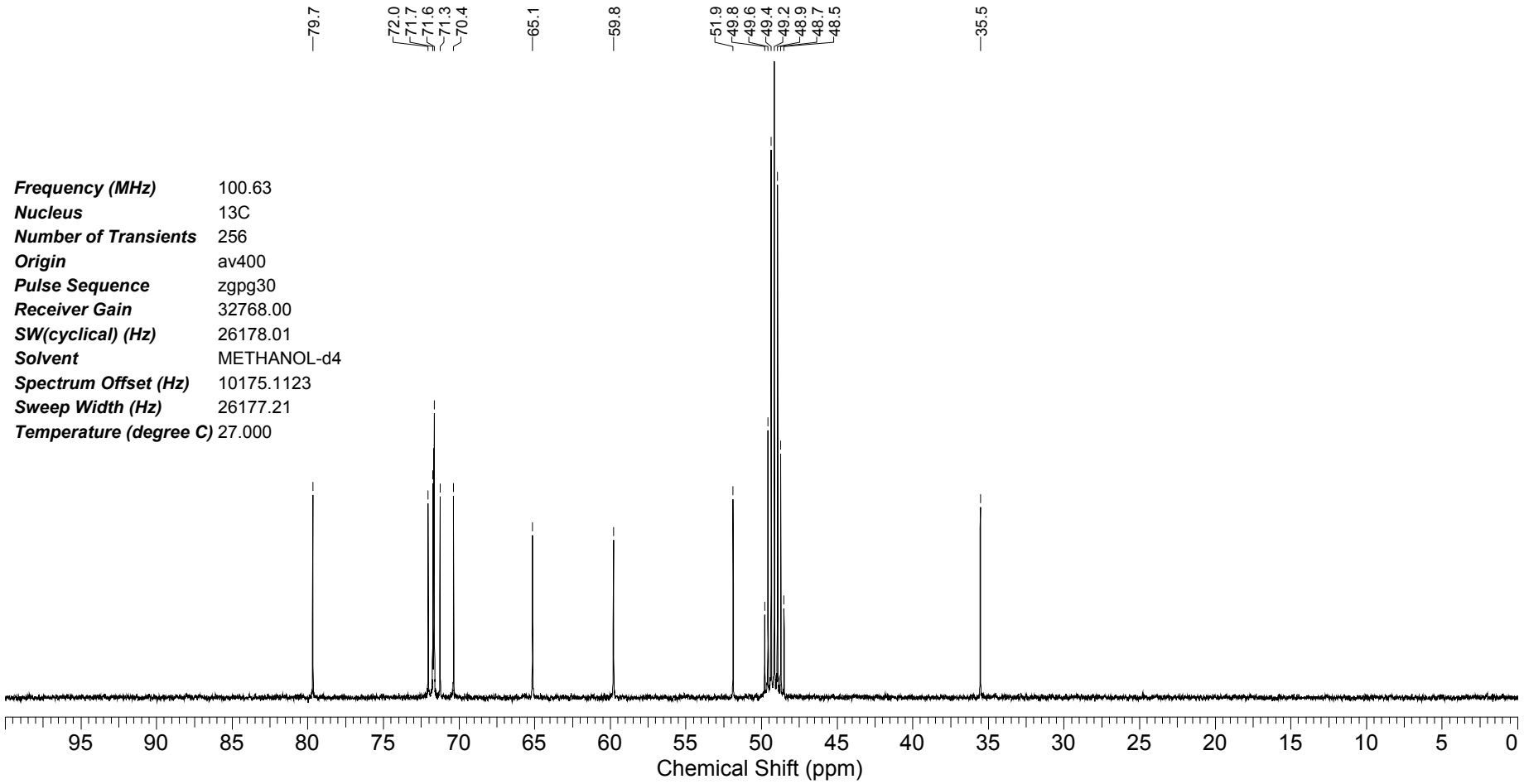


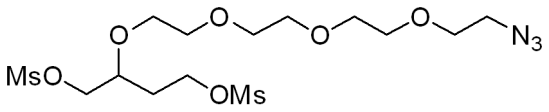




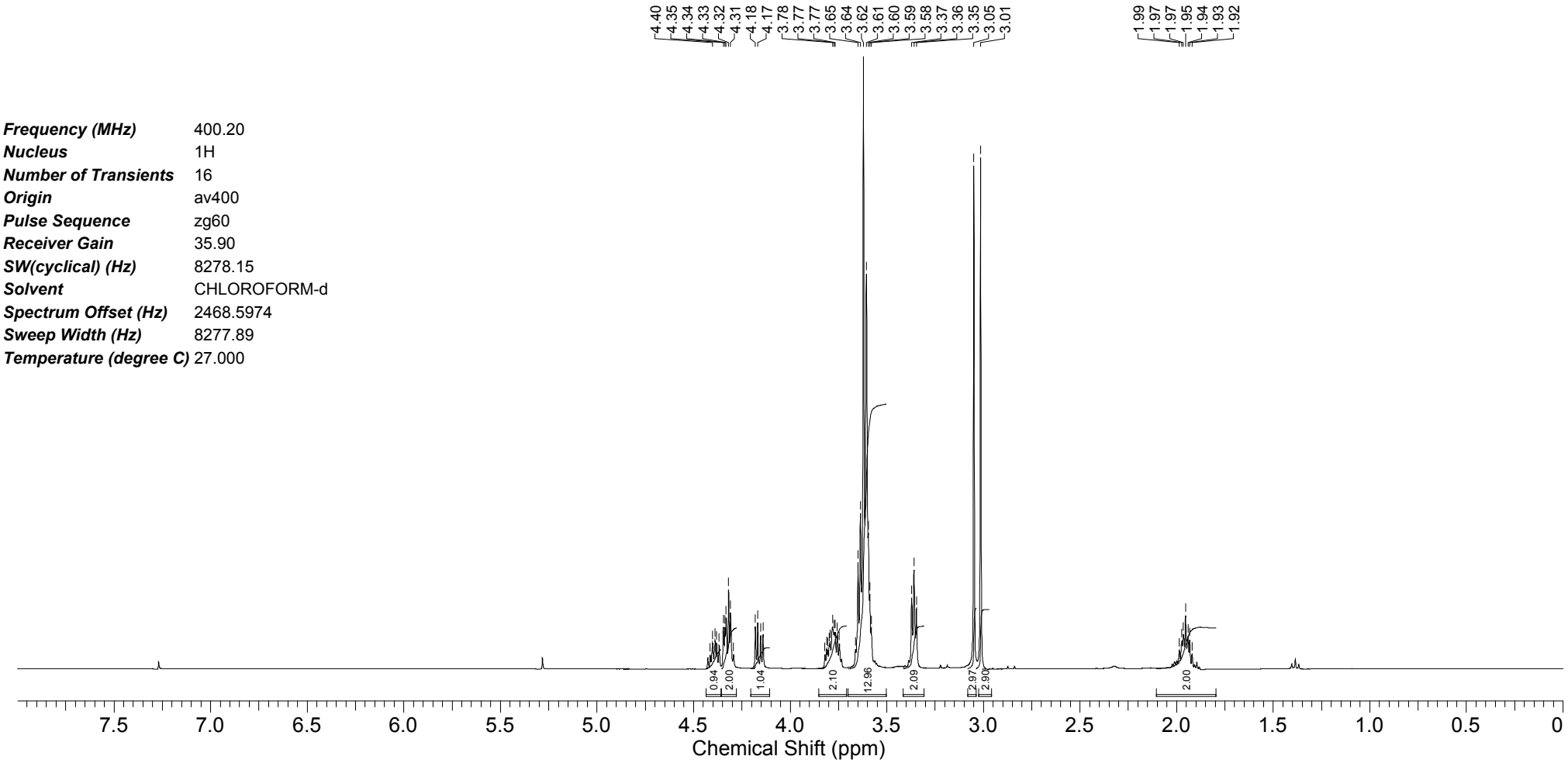
Frequency (MHz)	400.20
Nucleus	1H
Number of Transients	16
Origin	av400
Pulse Sequence	zg60
Receiver Gain	35.90
SW(cyclical) (Hz)	8278.15
Solvent	METHANOL-d4
Spectrum Offset (Hz)	2471.3989
Sweep Width (Hz)	8277.89
Temperature (degree C)	27.000

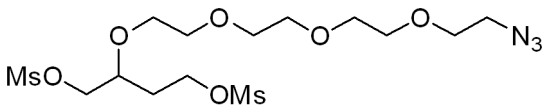


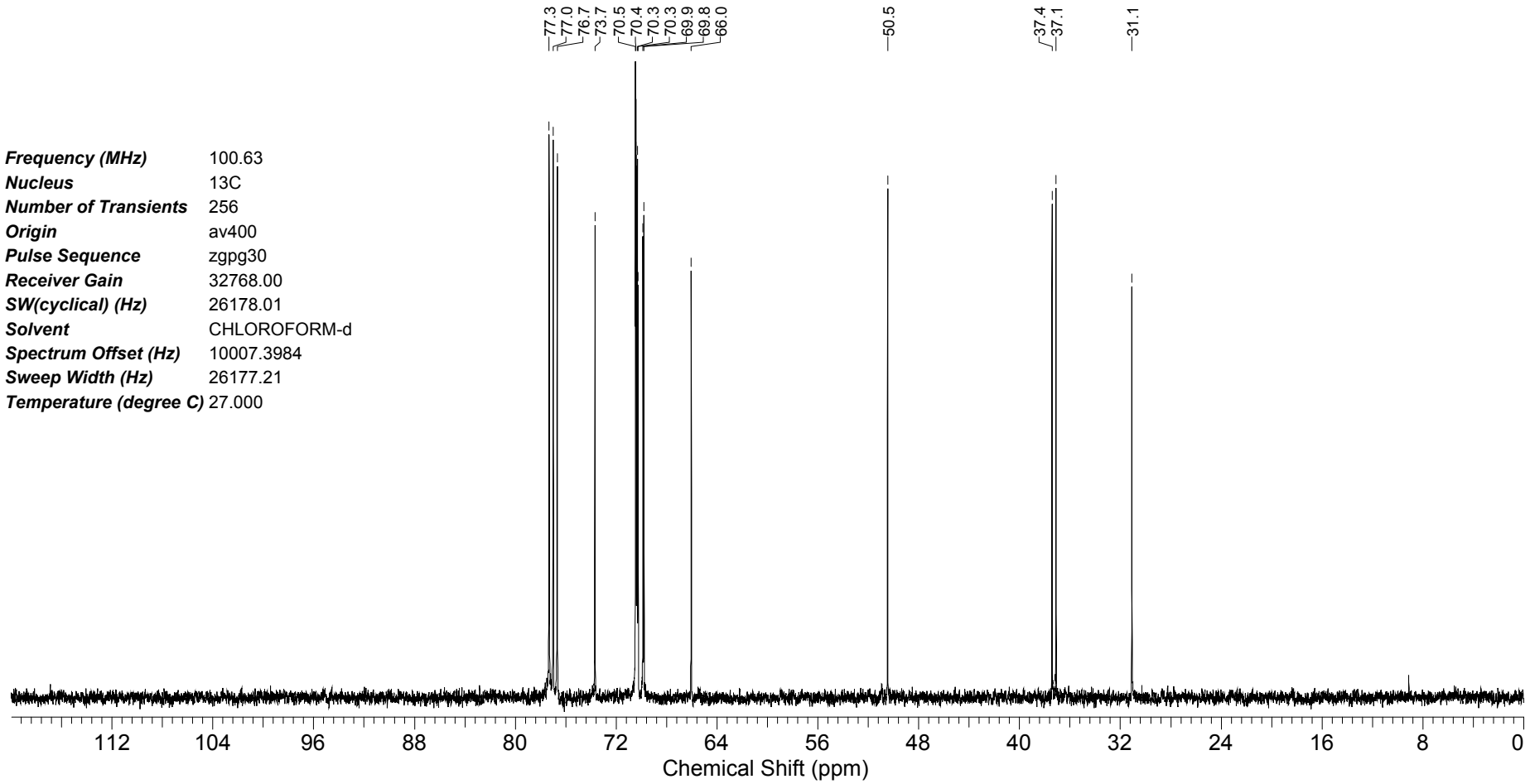




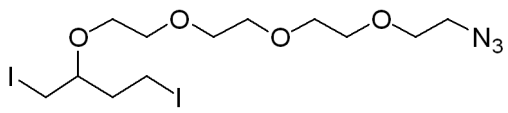
Frequency (MHz)	400.20
Nucleus	1H
Number of Transients	16
Origin	av400
Pulse Sequence	zg60
Receiver Gain	35.90
SW(cyclical) (Hz)	8278.15
Solvent	CHLOROFORM-d
Spectrum Offset (Hz)	2468.5974
Sweep Width (Hz)	8277.89
Temperature (degree C)	27.000



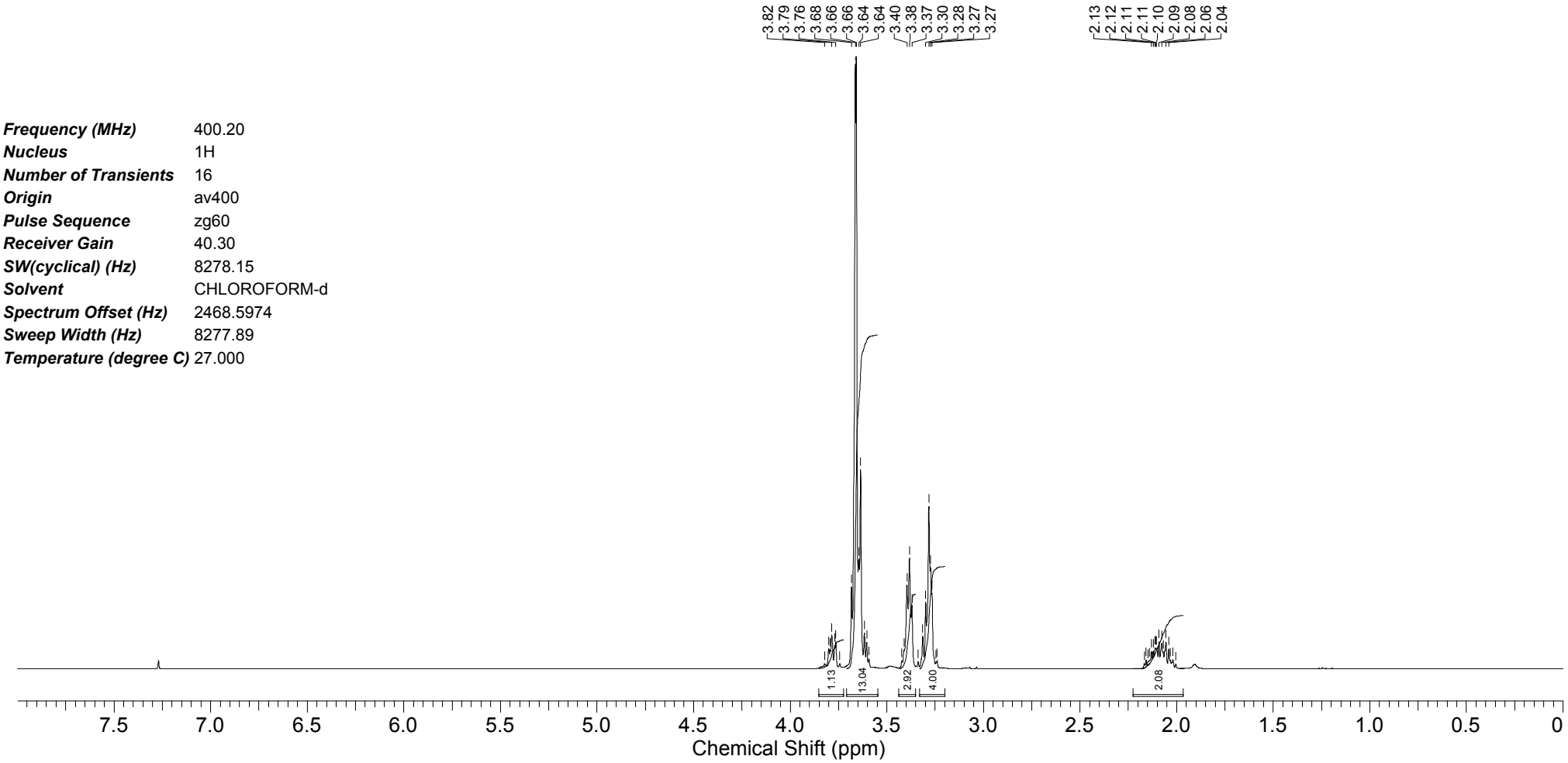


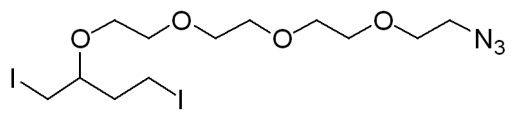


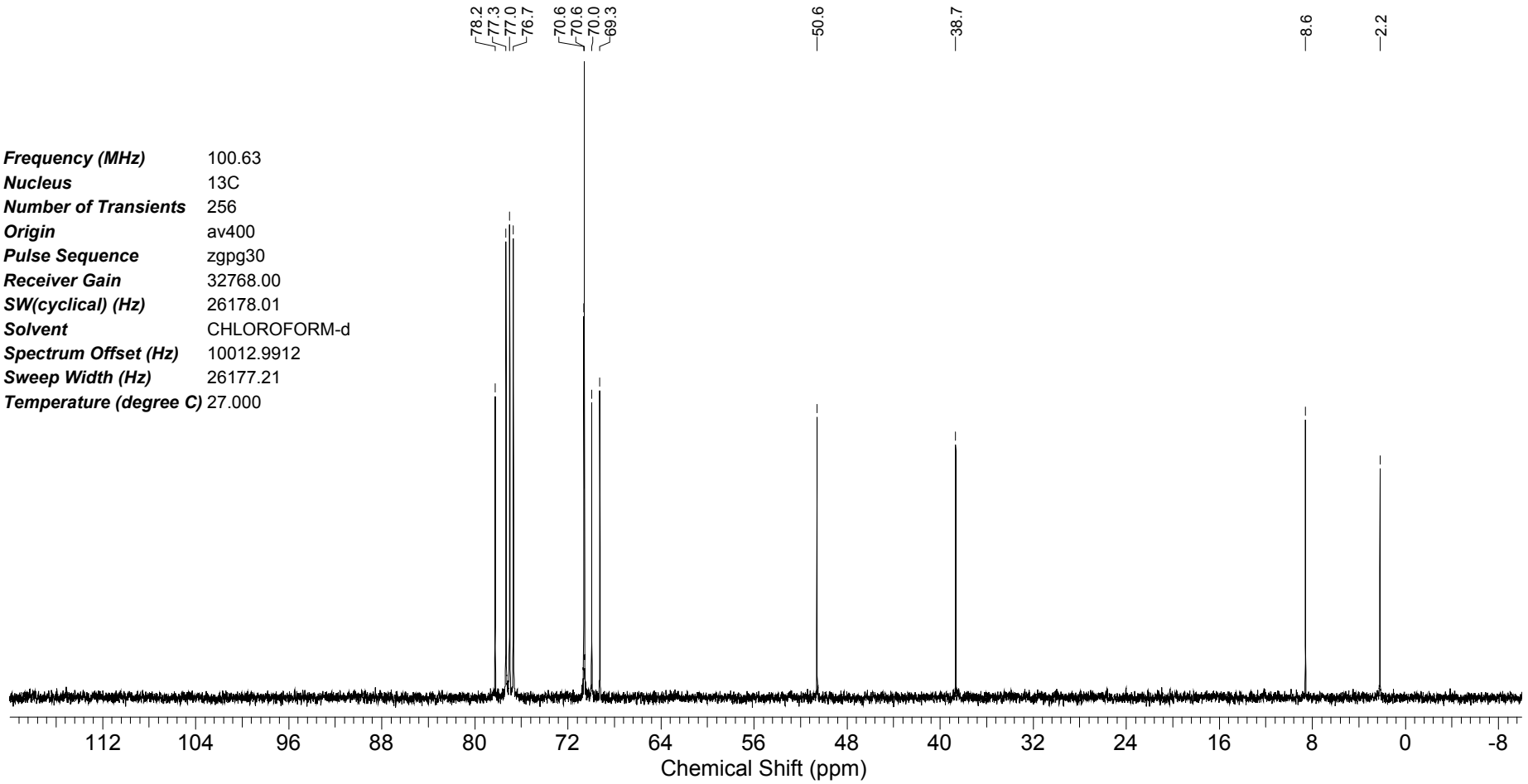
Frequency (MHz)	100.63
Nucleus	13C
Number of Transients	256
Origin	av400
Pulse Sequence	zgpg30
Receiver Gain	32768.00
SW(cyclical) (Hz)	26178.01
Solvent	CHLOROFORM-d
Spectrum Offset (Hz)	10007.3984
Sweep Width (Hz)	26177.21
Temperature (degree C)	27.000

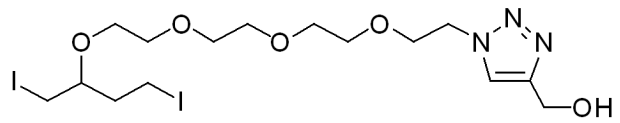


Frequency (MHz)	400.20
Nucleus	1H
Number of Transients	16
Origin	av400
Pulse Sequence	zg60
Receiver Gain	40.30
SW(cyclical) (Hz)	8278.15
Solvent	CHLOROFORM-d
Spectrum Offset (Hz)	2468.5974
Sweep Width (Hz)	8277.89
Temperature (degree C)	27.000

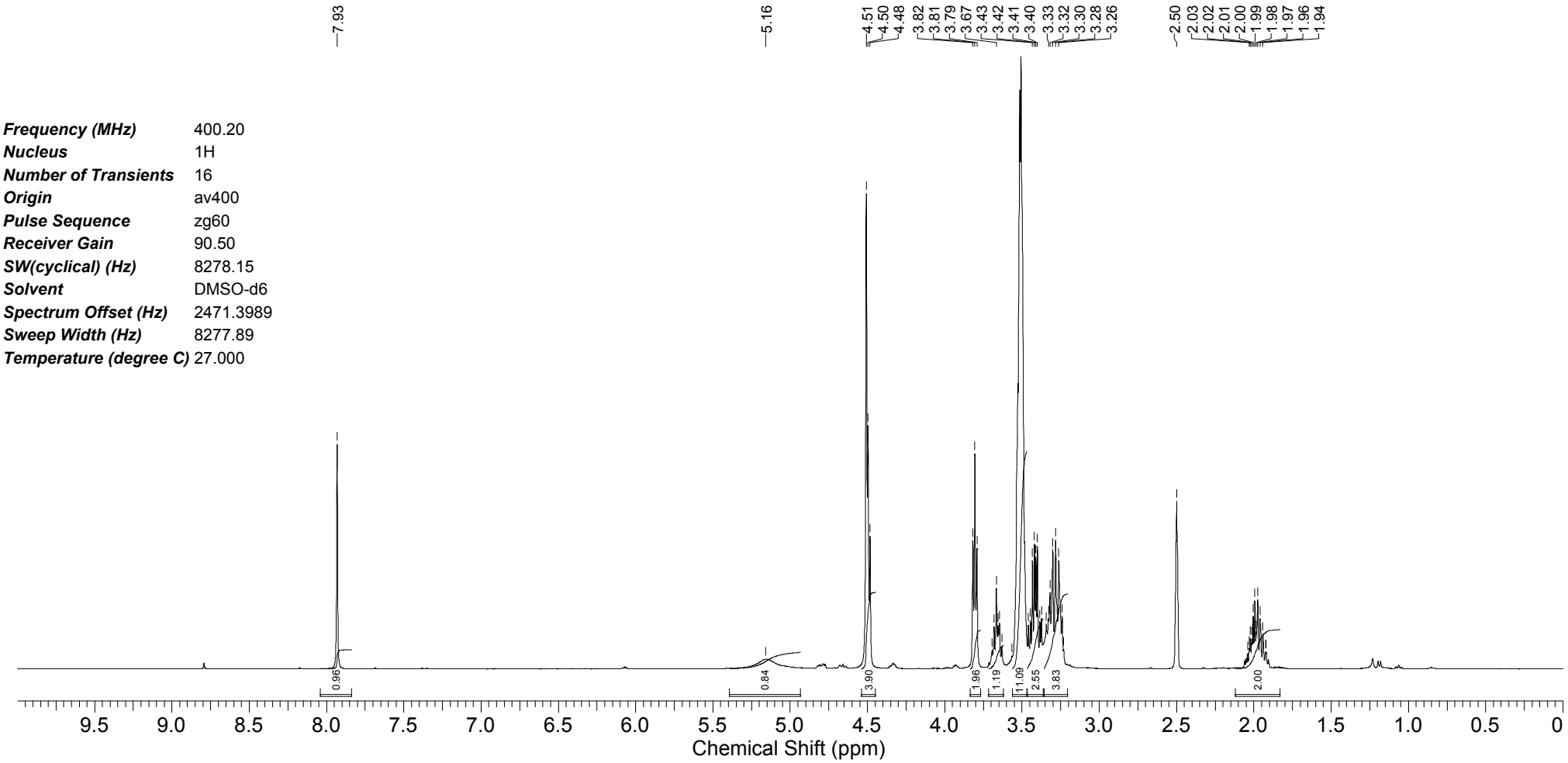


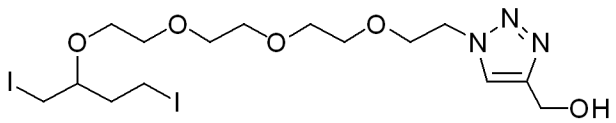


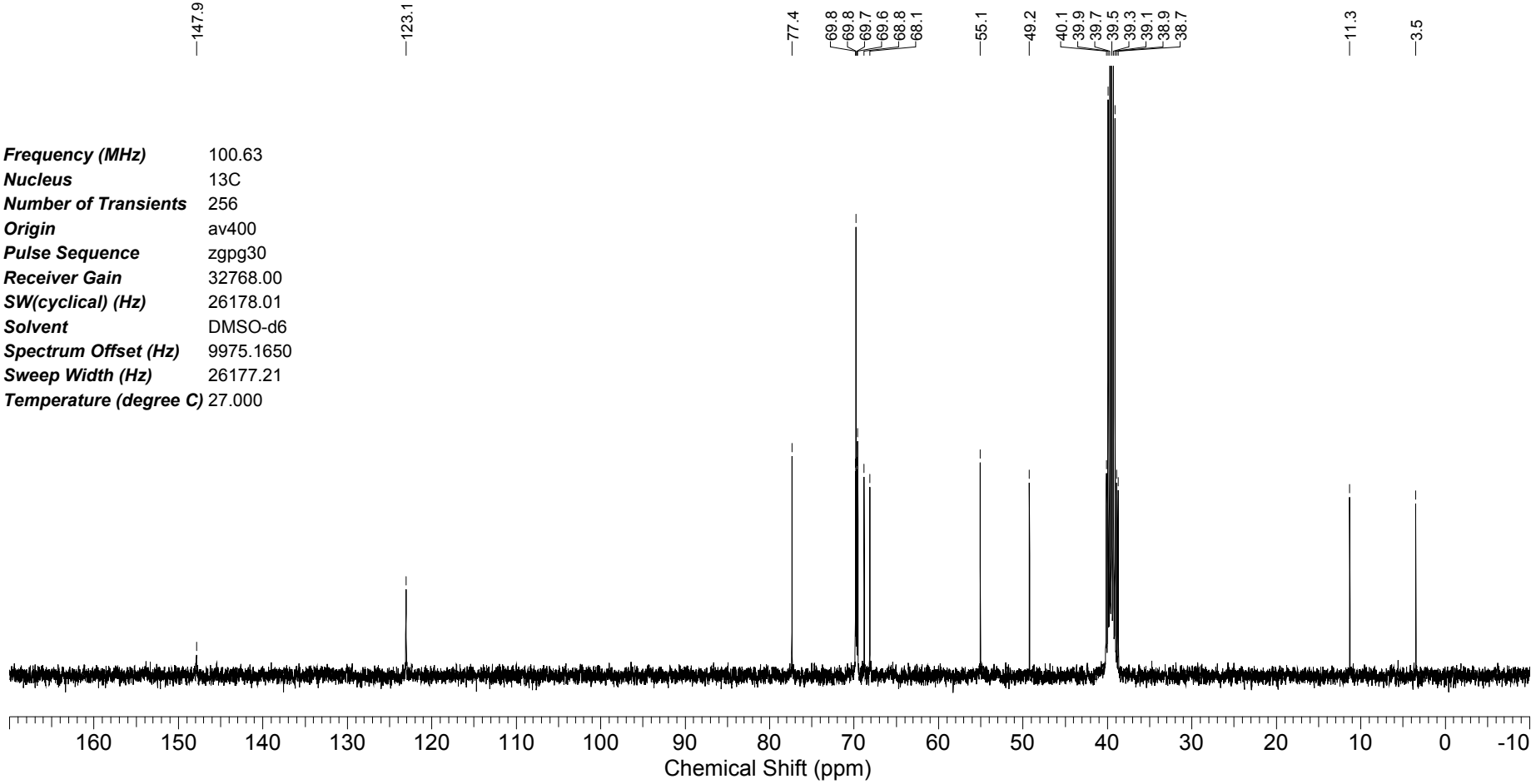




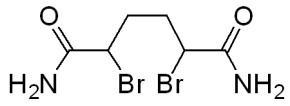
Frequency (MHz)	400.20
Nucleus	1H
Number of Transients	16
Origin	av400
Pulse Sequence	zg60
Receiver Gain	90.50
SW(cyclical) (Hz)	8278.15
Solvent	DMSO-d6
Spectrum Offset (Hz)	2471.3989
Sweep Width (Hz)	8277.89
Temperature (degree C)	27.000



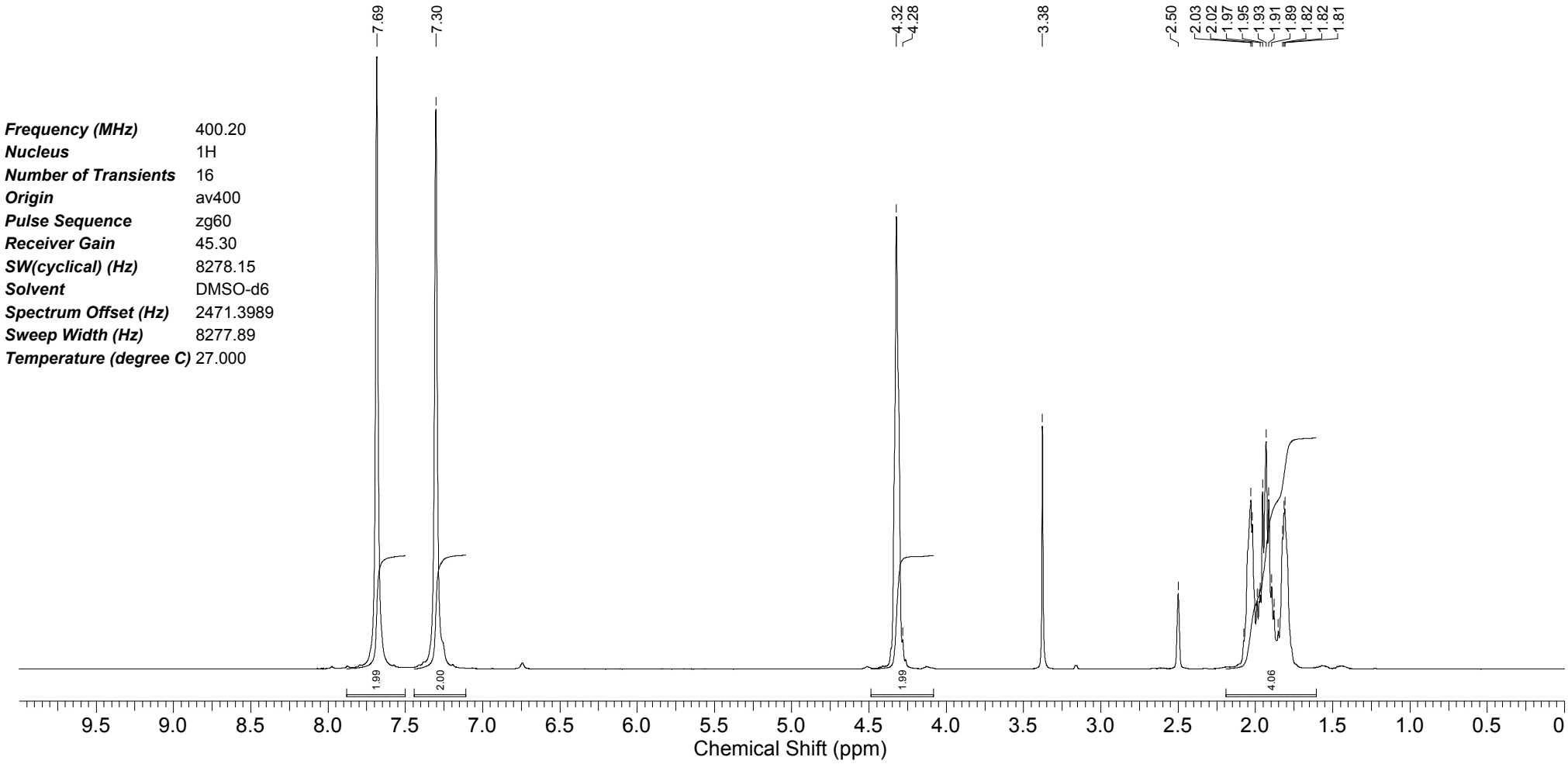




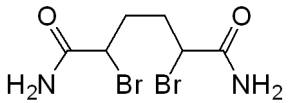
Frequency (MHz)	100.63
Nucleus	13C
Number of Transients	256
Origin	av400
Pulse Sequence	zgpg30
Receiver Gain	32768.00
SW(cyclical) (Hz)	26178.01
Solvent	DMSO-d6
Spectrum Offset (Hz)	9975.1650
Sweep Width (Hz)	26177.21
Temperature (degree C)	27.000



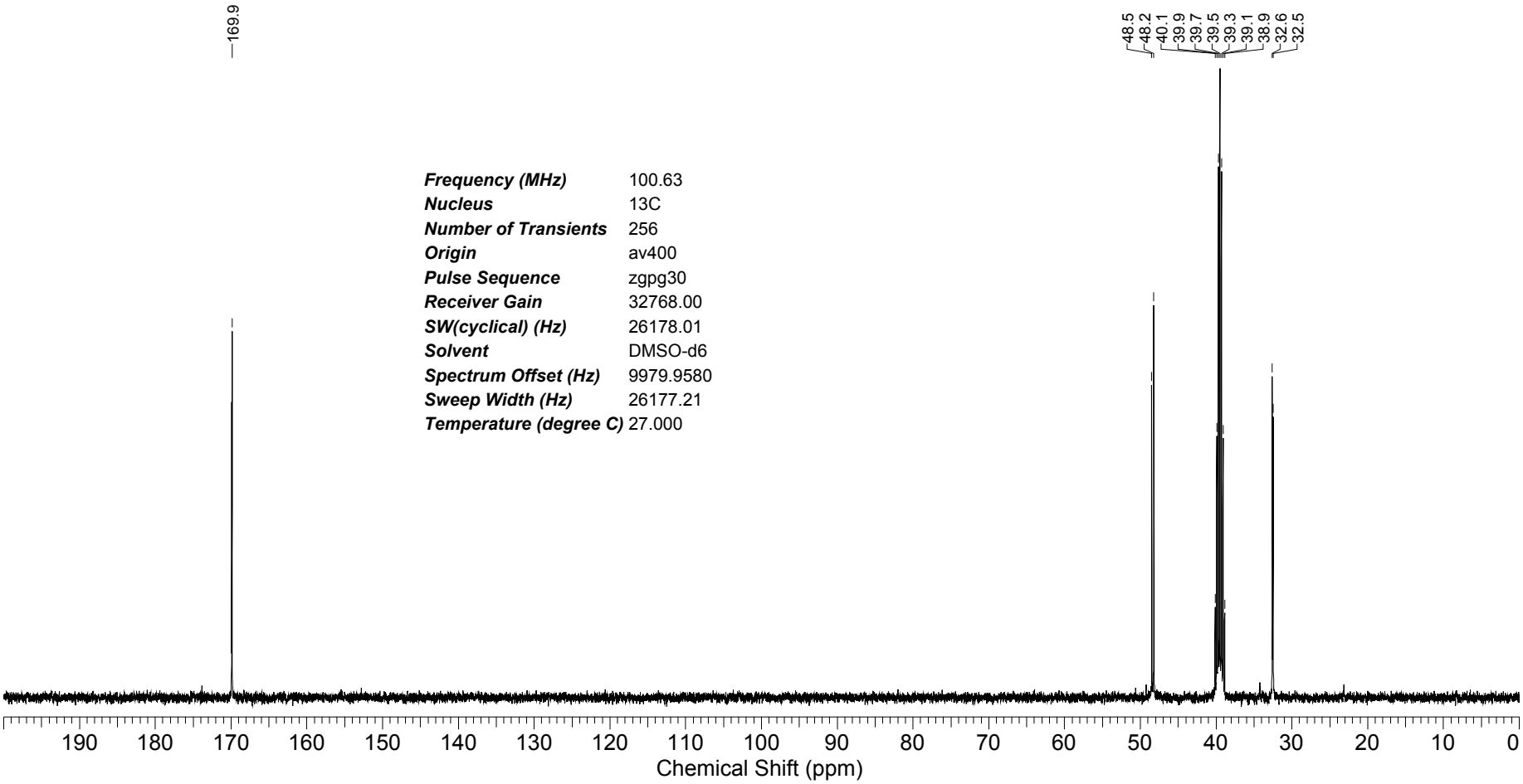
(meso, d/l)



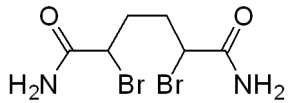
Frequency (MHz)	400.20
Nucleus	1H
Number of Transients	16
Origin	av400
Pulse Sequence	zg60
Receiver Gain	45.30
SW(cyclical) (Hz)	8278.15
Solvent	DMSO-d6
Spectrum Offset (Hz)	2471.3989
Sweep Width (Hz)	8277.89
Temperature (degree C)	27.000



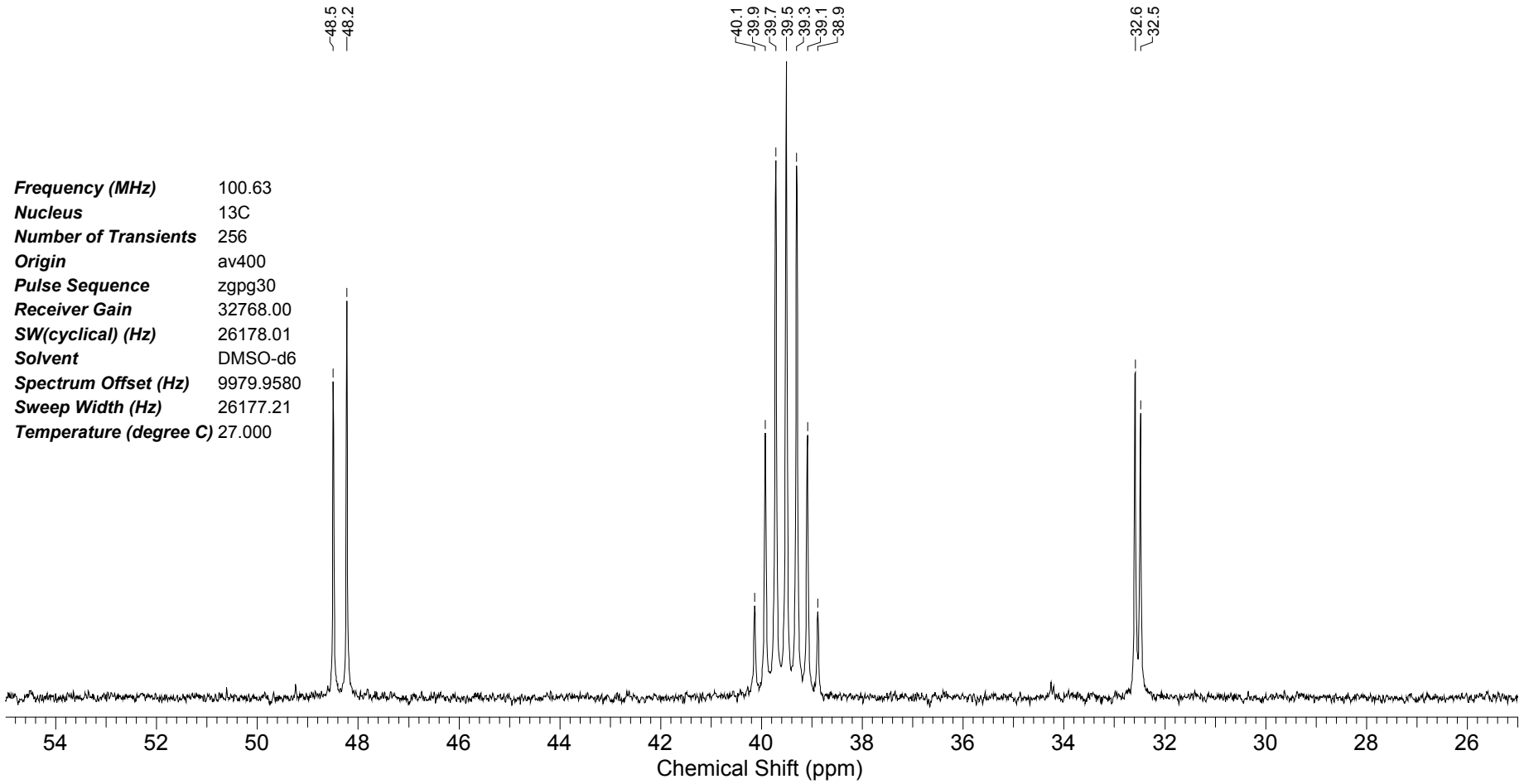
(meso, d/l)



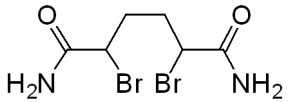
Frequency (MHz)	100.63
Nucleus	13C
Number of Transients	256
Origin	av400
Pulse Sequence	zgpg30
Receiver Gain	32768.00
SW(cyclical) (Hz)	26178.01
Solvent	DMSO-d6
Spectrum Offset (Hz)	9979.9580
Sweep Width (Hz)	26177.21
Temperature (degree C)	27.000



(meso, d/l)



Frequency (MHz)	100.63
Nucleus	13C
Number of Transients	256
Origin	av400
Pulse Sequence	zgpg30
Receiver Gain	32768.00
SW(cyclical) (Hz)	26178.01
Solvent	DMSO-d6
Spectrum Offset (Hz)	9979.9580
Sweep Width (Hz)	26177.21
Temperature (degree C)	27.000



(meso, d/l)

169.9
169.9

Frequency (MHz)	100.63
Nucleus	13C
Number of Transients	256
Origin	av400
Pulse Sequence	zgpg30
Receiver Gain	32768.00
SW(cyclical) (Hz)	26178.01
Solvent	DMSO-d6
Spectrum Offset (Hz)	9979.9580
Sweep Width (Hz)	26177.21
Temperature (degree C)	27.000