Regiodivergent Biocatalytic Hydroxylation of L-Glutamine Facilitated by Characterization of Non-Heme Dioxygenases from Non-Ribosomal Peptide Biosyntheses

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SUPPLEMENTARY MATERIAL

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General materials and methods

Unless otherwise noted, all chemicals and reagents for chemical reactions were purchased at the highest commercial quality and used without further purification. Reactions were monitored by thin layer chromatography (TLC) and liquid chromatography/mass spectrometry (LC/MS). TLC was performed with 0.25 mm E. Merck silica plates (60F-254) using short-wave UV light as the visualizing agent, and ninhydrin, $KMnO_4$, or phosphomolybdic acid and heat as developing agents. LC/MS was performed with Agilent 1260 Infinity System equipped with Poroshell 120 EC-C18 column (3.0 x 50 mm, 2.7 micron). C18 purification was performed with Biotage Isolera One equipped with Sfär C18 Duo column (60 g). NMR spectra were recorded on a Bruker spetrometer and calibrated using residual undeuterated solvent. Optical rotations were measured on Autopol IV polarimeter (Rudolph Research Analytical). Enzymes (DpnI, Q5 polymerase) were purchased from New England Biolabs (NEB, Ipswich, MA). pET28a(+)-Q4Ox and pET28a(+)-GloE expression vectors were obtained via DNA synthesis from Twist Bioscience and was used directly to transform electrocompetent *E. coli* strain BL21(DE3). Sonication was performed using a Qsonica Q500 sonicator. Purified enzymes were accessed via immobilized metal ion affinity chromatography with HisTrap HP column.

Protein and DNA sequences

Protein sequence of Q4Ox (Uniprot ID: I1R9B5)

MASNFTSIPVLDYPSSLSPSTKPAFLAELRDALVKVGFFQVRDPPIPLKLQQDALRLSAQFFDL
PTEKKLDIENVHSKRFLGYSRINSESTASGTDYLESILLGPNLPELGPEEPVYLHLQGPSQWPD
EVSVPGFRDVLESYHSQIQDFSIEFARLIAEALEMPLDTLTKLLGQPLFSRLKPTRYLPPSMNP
AAEDGSHGIGPHKDIAFMTYLLQGGTHNCLEVQNKLGHWVPVPPVPGALVVNIGRLLEVITGGV
CVATTHRVILKRQGFVDGDGKSLGPRISLPFFQFVNPRLTVDDVLVDVPHHIKDLVPDQVATTE
AETFFSGLFNNCIGDNIFVNHLTTYPRVGKRWYPDLMQLASEKQAESKRLDEQRRATEGHI

DNA sequence of FGSG_00048

ATGGCGTCGAACTTTACATCTATCCCGGTTCTGGACTATCCCTCTTCGTTGTCGCCTTCAACAA
AACCCGCCTTTTTGGCCGAGTTACGTGACGCGTTAGTCAAGGTTGGCTTTTTCCAGGTTCGCGA
TCCCCCTATCCCCTTGAAATTACAGCAAGATGCATTGCGCCTTTCTGCACAATTTTTCGACCTT
CCTACGGAGAAGAAGTTAGATATTGAGAACGTACACAGCAAACGTTTCCTTGGGTACTCCCGTA
TCAACAGTGAATCAACGGCGTCTGGGACTGACTACTTGGAAAGCATTCTGTTGGGACCGAATTT
ACCAGAGCTTGGTCCCGAGGAACCCGTCTACTTGCATCTTCAGGGGCCCTCACAATGGCCTGAC
GAGGTGTCGGTTCCCGGGTTTCGTGATGTGCTTGAGTCGTATCACAGCCAGATCCAGGACTTCT
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GCTTGGGCAGCCATTGTTTTCTCGCTTAAAGCCAACACGTTATTTACCCCCTTCTATGAATCCC
GCCGCAGAAGACGGATCACACGGCATCGGCCCCCACAAAGATATTGCCTTCATGACCTACCTTT
TACAGGGCGGGACACATAACTGCTTGGAAGTACAAAATAAACTGGGCCATTGGGTCCCGGTTCC
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GCTGAAACCTTCTTCAGTGGGTTATTCAACAACTGCATTGGAGACAACATTTTCGTCAACCATT
TAACCACGTATCCCCGTGTTGGTAAGCGCTGGTACCCAGATTTAATGCAACTGGCTTCGGAAAA
GCAGGCTGAGAGTAAACGTCTGGATGAGCAGCGCCGTGCCACCGAGGGACACATTTGA

Protein sequence of GloE (Uniprot ID: I1R9B5)

MDYIKQAESTQLSSLSLSRLEGNNAEESKRLLEACAQDGFFYLDLRDHKQLLVDYEALLEIIKQ
YFNEPLDQKMKDDRKSDTIGYEPVATSAGVLDGLPDYYESFKVSWNQLRDHVQELPTVVETNIE
VFDRFAKYVHSILLMILSRLSQTMGRNNDNRFESYHRDSIATRTNLTFLKYPKQDTTEHGVGHN
KHTDVGTLTFLLSGQRGLQRLTPEGWCHVEPRSGFAVVNVGDSLRFLSDCVLSSVIHRVLPVGA
HQTEDRYTLAYFLRPEDDAVFKDINGNLVSARSWHDRKFDHFRASHNQQKNDTILMGGMEENQK
FLQYKFQA

DNA sequence of GloE

ATGGATTACATTAAACAGGCTGAATCTACCCAGCTGTCTTCTCTGAGCTTGTCTCGCTTGGAAG
GAAATAACGCCGAAGAGTCAAAACGTCTTTTGGAAGCCTGCGCTCAGGACGGCTTCTTCTATTT
GGACTTACGTGACCATAAACAGTTACTGGTTGACTATGAAGCCTTGCTGGAAATCATCAAGCAA
TACTTCAACGAACCTCTGGACCAAAAGATGAAGGACGATCGTAAGAGTGATACGATCGGCTATG
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TACAAACTTGACCTTTCTTAAGTATCCCAAGCAAGACACGACTGAACATGGAGTTGGTCACAAT
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CACCAAACCGAAGATCGCTATACGTTAGCTTACTTTCTTCGTCCAGAAGACGACGCCGTTTTTA
AGGACATCAATGGTAACCTGGTGTCCGCGCGCAGTTGGCACGACCGCAAATTCGACCATTTCCG
CGCTAGTCACAATCAACAGAAGAATGATACCATTCTTATGGGGGGTATGGAGGAGAACCAGAAA
TTCTTACAATACAAGTTTCAAGCCTGA

For the construction of expression vector, each of the above sequences was inserted between the NdeI and BamHI restriction sites within the commercial pET28a(+) vector. The resulting expression vector was used directly to transform electrocompetent *E. coli* strain BL21(DE3). Variants were stored as glycerol stocks at –80 ºC.

General procedure for amino acid purification with Dowex 50WX8 cation exchange resin

Dowex 50WX8 resin (20 mg/1 μmol of amino acid) was slurry-packed with 1 M NH_4OH in a flash chromatography column, and then washed with ~1 column volume (CV) of 1 M NH_4OH, and then ~ 2 CV of H_2O. Next, the resin was washed with 1 M HCl until pH = 1, and then with H_2O until pH = 6–7. The acidified crude reaction mixture was loaded directly onto the column, washing with H_2O until pH = 7, and with 1 M NH_4OH until product was no longer eluting from the column. Amino acid containing fractions were pooled, concentrated *in vacuo*.

General procedure for enzyme expression and purification

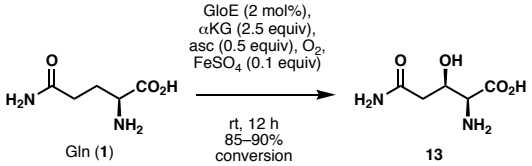
2 x 250 mL TB_{kan} was inoculated with an overnight culture (1 mL, LB_{kan}) of recombinant *E. coli* BL21(DE3) cells harboring a pET28a(+) plasmid encoding the appropriate enzyme (Q4Ox or GloE). The cultures were shaken at 250 rpm at 37 °C for roughly 2.5 h or until an optical density of 0.7–1.0 was reached. Cultures were cooled on ice for 20 min and then induced by adding IPTG to a final concentration of 25 μM. The cultures were allowed to continue for another 20 hours at 20 °C and shaking at 250 rpm. Cell were harvested by centrifugation (4 °C, 15 min, 3,000xg), and the cell pellet was stored at –20 °C or below for at least 2 h. Purification was performed with an AKTA pure FPLC system (GE Healthcare). The thawed cell pellet was resuspended in Ni-NTA buffer A (25 mM Tris•HCl, 200 mM NaCl, 25 mM imidazole, pH 9.0, 4 mL/g of cell wet weight) and lysed by sonication (3x1 min, 50% duty cycle). The lysate was centrifuged at 15,000xg for 30 min at 4 °C to remove cell debris. The collected supernatant was subjected to a Ni-NTA

chromatography step using a Ni Sepharose column (HisTrap-HP, GE healthcare, Piscataway, NJ). The protein was eluted from the Ni Sepharose column using 25 mM Tris•HCl, 200 mM NaCl, 300 mM imidazole, pH 8.0. Ni-purified protein was buffer exchanged into 0.05 M phosphate buffer (pH = 8.0) using a 30 kDa MW cut-off centrifugal filter. Protein concentrations were determined by A280 with calculated extinction coefficients as obtained at https://web.expasy.org/protparam. For storage, proteins were portioned into 100 µL aliquots, flash frozen on liquid N_2, and stored at –80 °C.

Total turnover number (TTN) measurements

A 20 ml scintillation vial was charged with the appropriate amino acid (80 µmol, 1.0 equiv, 20 mM final concentration), L-ascorbic acid (40 µmol, 0.50 equiv, 10 mM final concentration), and α-ketoglutaric acid (disodium salt dihydrate, 200 µmol, 2.5 equiv, 50 mM final concentration). 50 mM KPi buffer was added to the vial (pH 8.0, 4.0 mL), followed by 40 µL of $FeSO_4$ solution in H2O (200 mM, 0.10 equiv, 2 mM final concentration). The reaction was started by the addition of the appropriate enzyme stock solution (final concentration of Q4Ox = 2 µM, 0.00010 equiv, final concentration of GloE = 0.40 mM, 0.02 equiv) and shaken for 12 h at 20 ºC, 250 rpm under air. After quenching with 1.0 mL of 1 M HCl, the crude reaction mixture was centrifuged (4,000 rpm, 5 min) and the supernatant was submitted directly to ion exchange purification on Dowex 50WX8 resin. For Q4Ox, yield of hydroxylation product were determined using ^{1}H NMR analysis employing ethylene carbonate as an internal standard. TTN was calculated by dividing the percentage yield of hydroxylation product with the mol% loading of enzyme. For GloE, reaction conversion was determined using ^{1}H NMR analysis by obtaining the ratio of hydroxylated product: unreacted starting material.

Characterization of 3-OH-Gln product from GloE reaction



The above reaction with GloE provided ca. 1.0:0.15 ratio of **13**:**1** based on ^{1}H NMR analysis after ion exchange purification on Dowex 50WX8 resin. This ^{1}H NMR spectrum was sufficiently clean

to allow identification and characterization of the C3 hydroxylated product, which matches spectral data previously reported in the literature.[1,2]

^{1}H NMR (400 MHz, Deuterium Oxide): δ 4.35 – 4.27 (m, 1H), 3.45 (d, *J* = 4.8 Hz, 1H), 2.58 (dd, *J* = 14.8, 3.9 Hz, 1H), 2.50 (dd, *J* = 14.8, 9.5 Hz, 1H).

Determination of Michaelis-Menten parameters of Gln hydroxylation with Q4Ox

Kinetic parameters were determined by incubating 3 μM Q4Ox with 25 mM αKG, 25 mM L-ascorbic acid, 1 mM $FeSO_4$ and various concentrations of L-glutamine (0.50 mM to 4.0 mM) in 50 mM kPi buffer (pH 8.0, 2.5 mL total reaction volume). After 30 s, 1 min and 2 min interval, a 200 μL aliquot of the reaction was sampled and quenched with 200 μL of 50 mM Fmoc-OSu solution in MeCN and 100 μL of sat. aq. $NaHCO_3$. After 15 min of incubation, 100 μL of 30 mM $TsNH_2$ was added as an internal standard for quantitation. The samples were centrifuged at 15,000 rpm for 3 min and analyzed by LCMS using the extracted ion chromatogram (EIC) mode, calibrating against standard curves made independently from Fmoc-derivatized product standards and $TsNH_2$. Apparent kinetic parameters were calculated from a nonlinear fit of the initial velocity data to the Michaelis-Menten equation with the KaleidaGraph data analysis software (Synergy). The error bars in the plots represent the standard deviations calculated from replicate measurements and the errors for the fitted parameters are standard errors for the fits.

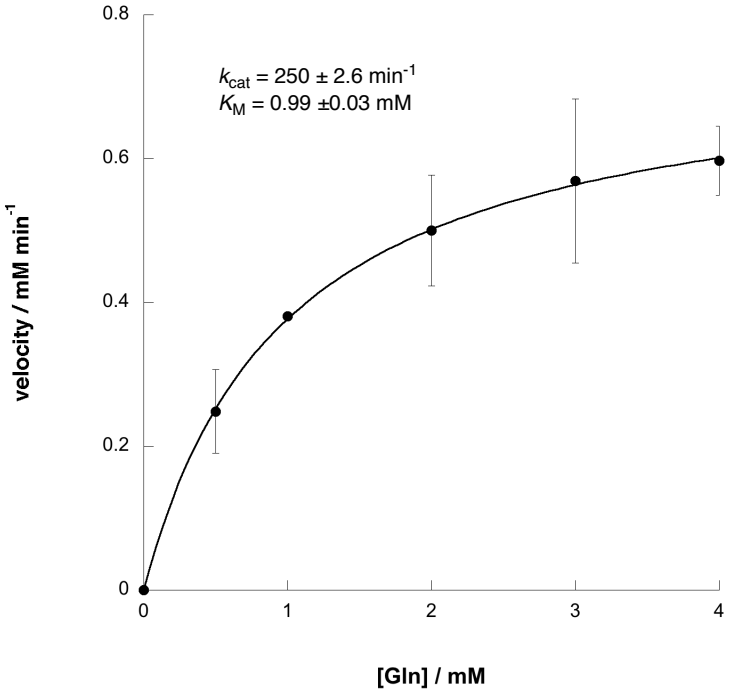
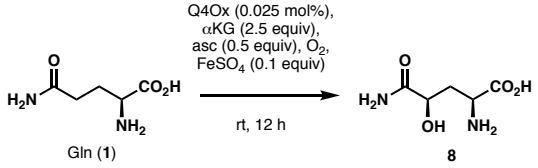


Figure S1. Michaelis-Menten plot of Gln hydroxylation with Q4Ox.

Synthetic procedure

Large-scale hydroxylation of glutamine with purified Q4Ox



A 250 ml Erlenmeyer flask was charged with L-glutamine (105 mg, 0.72 mmol, 1.0 equiv, 20 mM final concentration), L-ascorbic acid (63 mg, 0.36 mmol, 0.50 equiv, 10 mM final concentration), and α-ketoglutaric acid (disodium salt dihydrate, 407 mg, 1.80 mmol, 2.5 equiv, 50 mM final concentration) and $FeSO_4$ (heptahydrate, 20 mg, 0.072 mmol, 0.1 equiv, 2 mM final concentration), followed by 50 mM KPi buffer (pH 8.0, 36 mL). The reaction was started by the addition of Q4Ox stock solution (final concentration = 5 μM, 0.00025 equiv) and shaken for 12 h at 20 ºC, 250 rpm under air. After quenching with 7.2 mL of 1 M HCl, the crude reaction mixture was centrifuged (4,000 rpm, 5 min). The supernatant was collected and following resuspension of the residual pellet in 20 mL of 0.2 M HCl, the crude suspension was centrifuged (4,000 rpm, 5 min) and the supernatant was collected. The combined supernatant was submitted directly to ion exchange purification on Dowex 50WX8 resin. Amino acid-containing fractions were combined, concentrated in vacuo and lyophilized to afford 99 mg of **8** as dark yellow powder which was determined to be >95% pure by ^{1}H NMR analysis.

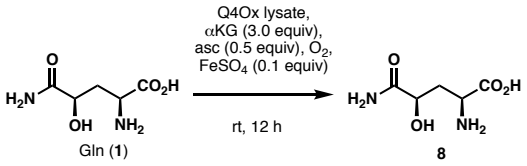
^{1}H NMR (400 MHz, Deuterium Oxide): δ 4.29 (dd, *J* = 10.3, 3.5 Hz, 1H), 4.14 (dd, *J* = 6.8, 5.9 Hz, 1H), 2.37 (ddd, *J* = 15.0, 5.9, 3.4 Hz, 1H), 2.00 (ddd, *J* = 15.1, 10.4, 6.8 Hz, 1H).

^{13}C NMR (101 MHz, Deuterium Oxide): δ 178.2, 171.1, 162.4 (q, *J* = 36.5 Hz), 115.9 (q, *J* = 290.5 Hz), 68.5, 50.8, 33.2.

$[\alpha]_D^{23}$ = +18.8º (c = 0.5, H_2O)

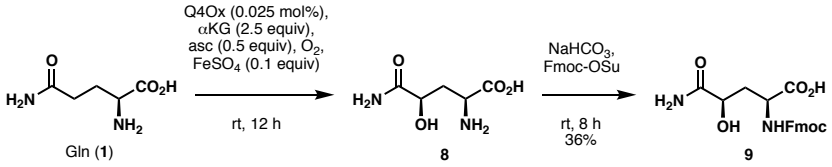
HRMS (ESI-TOF): calcd for $C_5H_{11}N_2O_4^+$ ($[M+H]^+$) 163.0713, found 163.0744.

Large-scale hydroxylation of glutamine with lysate of E. coli expressing Q4Ox



200 mL TB_{kan} was inoculated with an overnight culture (1 mL, LB_{kan}) of recombinant *E. coli* BL21(DE3) cells harboring a pET28a(+) plasmid encoding for Q4Ox. The cultures were shaken at 250 rpm at 37 °C for roughly 2.5 h or until an optical density of 0.7–1.0 was reached. Cultures were cooled on ice for 20 min and then induced by adding IPTG to a final concentration of 25 μM. The cultures were allowed to continue for another 20 hours at 20 °C and shaking at 250 rpm. Cell were harvested by centrifugation (4 °C, 15 min, 4,000xg) and resuspended in pH 8 KPi (50 mM) to a final OD_{600} of 20. This procedure reliably produces ca. 70 mL of cell suspension. Cells were lysed by sonication (50% amplitude, 1 s on, 4 s off for 3 minutes) and the suspension was centrifuged (4 °C, 15 min, 4,000xg). A 250 mL Erlenmeyer flask was charged with Gln (204 mg, 1.40 mmol, 1.0 equiv, 20 mM final concentration), L-ascorbic acid (123 mg, 0.70 mmol, 0.50 equiv, 10 mM final concentration), α-ketoglutaric acid (disodium salt dihydrate, 949 mg, 4.20 mmol, 3.0 equiv, 60 mM final concentration) and $FeSO_4$ (heptahydrate, 39 mg, 0.14 mmol, 0.10 equiv, 2 mM final concentration), followed by 70 mL of clarified cell lysate. The reaction was left shaking at 250 rpm, 20 ºC overnight. After quenching with 15 mL of 1 M HCl, the crude reaction mixture was centrifuged (4,000 rpm, 5 min). The supernatant was collected and following resuspension of the residual pellet in 20 mL of 0.2 M HCl, the crude suspension was centrifuged (4,000 rpm, 5 min) and the supernatant was collected. The combined supernatant was submitted directly to ion exchange purification on Dowex 50WX8 resin. Amino acid-containing fractions were combined, concentrated in vacuo and lyophilized to afford 174 mg of **8** as dark yellow powder which was determined to be >95% pure by ^{1}H NMR analysis.

Telescoped procedure for one-pot Fmoc protection of 4-OH-Gln



Large scale hydroxylation was performed following the above procedure with purified Q4Ox. After 12 h of shaking, 9 mL of sat aq. $NaHCO_3$ was added, followed by a solution of Fmoc-Osu (485 mg, 1.44 mmol, 2.0 equiv) in 36 mL of MeCN. The mixture was shaken for 8 h at room temperature and then charged with 30 mL of MeOH. The resulting mixture was centrifuged (4,000 rpm, 5 min) and the supernatant was collected. The residual pellet was resuspended in 1:1 mixture of MeCN:MeOH (30 mL). The resulting suspension was centrifuged (4,000 rpm, 5 min) and the

supernatant was collected. The combined supernatant was concentrated in vacuo until a residual volume of ~ 5 mL was obtained and then purified on a C18 reverse phase column (Biotage Sfär Duo, 60 g) with MeCN/H_2O as the eluent system (1% MeCN/H_2O to 100% MeCN over 10 CV) to afford 99 mg of **9** as an off-white solid.

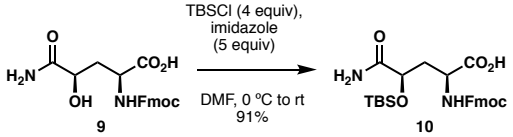
^{1}H NMR (400 MHz, Methanol-*d*$_4$): δ 7.79 (dd, *J* = 7.5, 0.9 Hz, 2H), 7.68 (dd, *J* = 7.6, 2.9 Hz, 2H), 7.39 (td, *J* = 7.5, 1.1 Hz, 2H), 7.34 – 7.23 (m, 2H), 4.45 (t, *J* = 6.7 Hz, 1H), 4.33 (qd, *J* = 10.4, 7.1 Hz, 1H), 4.23 (t, *J* = 7.1 Hz, 1H), 4.19 (dd, *J* = 8.0, 4.5 Hz, 1H), 2.34 (ddd, *J* = 14.1, 6.6, 4.5 Hz, 1H), 2.01 (ddd, *J* = 14.5, 8.1, 6.9 Hz, 1H).

^{13}C NMR (101 MHz, Methanol-*d*$_4$): δ 179.8, 175.6, 158.4, 145.3, 145.3, 142.6, 128.8, 128.2, 126.3, 120.9, 69.9, 68.1, 52.3, 37.6.

$[\alpha]_D^{23}$ = +3.1º (c = 1.0, MeOH)

HRMS (ESI-TOF): calcd for $C_{20}H_{20}N_2NaO_6^+$ ([M+Na]$^+$) 407.1214, found 407.1217.

TBS protection of Fmoc 4-OH-Gln



To a solution of **9** (45 mg, 0.117 mmol, 1.0 equiv) in 2 ml of DMF was sequentially added imidazole (40 mg, 0.585 mmol, 5.0 equiv) and TBSCl (71 mg, 0.468 mmol, 4.0 equiv) at 0 ºC. The mixture was stirred at 0 ºC to rt overnight and quenched by 1 M HCl until pH ~2–3. The aqueous layer was extracted with EtOAc (3 x 15 mL) and the combined organic extracts were washed with H_2O (10 mL) and sat aq. NaCl (3 x 10 mL), dried over Na_2SO_4 and concentrated in vacuo. Purification by silica gel chromatography (5:1 hexanes:EtOAc with 0.5% AcOH to 1:1 hexanes:EtOAc with 0.5% AcOH) gave **10** as a white foam (53 mg, 91%).

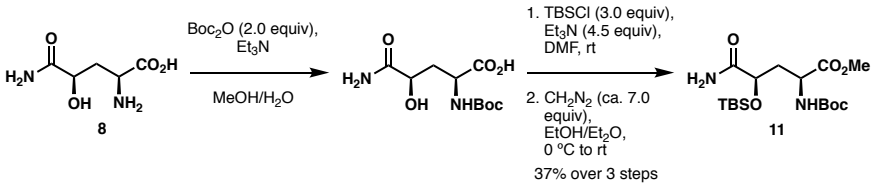
^{1}H NMR (400 MHz, Methanol-*d*$_4$): δ 7.79 (d, *J* = 7.4 Hz, 2H), 7.68 (d, *J* = 7.5 Hz, 2H), 7.38 (t, *J* = 7.4 Hz, 2H), 7.31 (tt, *J* = 7.4, 1.5 Hz, 2H), 4.50 (brs, 1H), 4.37 – 4.25(m, 3H), 4.21 (t, *J* = 6.9 Hz, 1H), 2.41 – 2.23 (m, 1H), 2.22 – 2.11 (m, 1H), 0.96 (s, 9H), 0.15 (d, *J* = 11.3 Hz, 6H).

^{13}C NMR (101 MHz, Methanol-*d*$_4$): δ 145.2, 142.5, 128.8, 128.2, 126.3, 120.9, 72.0, 68.1, 38.1, 30.7, 26.27, 19.0, -4.9, -5.2.

$[\alpha]_D^{23}$ = +1.5º (c = 1.0, MeOH)

HRMS (ESI-TOF): calcd for $C_{26}H_{35}N_2O_6Si^+$ ([M+H]$^+$) 499.2259, found 499.2278.

Synthesis of compound 11



A previous procedure for the derivatization of 4-OH-Cit was adapted for this synthesis.[3] To a suspension of 4-OH-Gln (89 mg, 0.55 mmol, 1.0 equiv, obtained from reaction with purified Q4Ox) in 4:1 MeOH:H_2O (4 ml) was added Et_3N (0.4 mL). Boc_2O (240 mg, 1.10 mmol, 2.0 equiv) was added in a single portion and the mixture was stirred at room temperature for 16 h. The mixture was concentrated *in vacuo* and azeotroped a few times with toluene to remove residual water and the resulting crude material was taken to the next step without further purifications.

The crude material was dissolved in DMF (1.5 mL), followed by addition of Et_3N (0.34 mL, 2.48 mmol, 4.5 equiv) and TBSCl (249 mg, 1.65 mmol, 3.0 equiv). After stirring for 24 h, EtOAc (10 mL) and H_2O (1.5 mL) were added, followed by 1 M HCl until a pH of 1–2 was reached. The aqueous layer was extracted with EtOAc (2 x 10 mL) and the combined organic extracts were washed with H_2O (5 mL) and sat aq. NaCl (2 x 10 mL), dried over Na_2SO_4 and concentrated in vacuo.

Diazomethane was generated from N-Me-nitroso-urea (400 mg, 3.88 mmol) following a previously reported procedure.[4] The crude acid was dissolved in Et_2O (5.0 mL) and EtOH (0.5 mL) (0.1 M final concentration) and the resulting solution was cooled to 0 °C. Diazomethane (in ~ 10 mL Et_2O, stored at 0 °C) was added portion-wise and the resulting mixture was allowed to stir at 0 °C for 2 h, then at room temperature for 3 h. AcOH was added dropwise until the reaction went from yellow to colorless. The solution was subsequently concentrated in vacuo and the crude material was purified by silica gel chromatography (gradient: 1:4 EtOAc:hexanes to 3:7 EtOAc:hexanes) to provide 79 mg of **11** as a white foam with minor TBS–OH impurities.

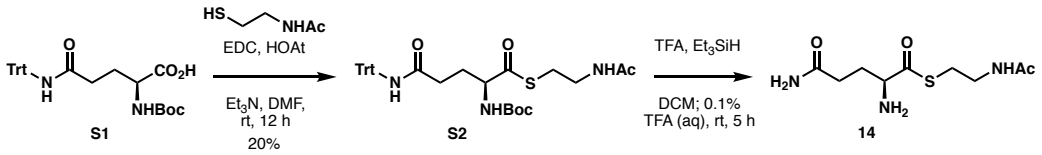
^{1}H NMR (400 MHz, Methanol-d_4): δ 6.56 (s, 1H), 4.36 – 4.27 (m, 1H), 4.24 (q, J = 5.3, 4.5 Hz, 1H), 3.72 (s, 3H), 2.25 – 2.04 (m, 2H), 0.96 (s, 9H), 0.16 (d, J = 16.9 Hz, 6H).

^{13}C NMR (101 MHz, Methanol-d_4): δ 181.7, 174.6, 157.9, 80.8, 72.5, 52.8, 51.3, 37.4, 28.7, 26.3, 18.9, -4.8, -5.1.

$[\alpha]_D^{23}$ = –4.7° (c = 1.0, MeOH)

HRMS (ESI-TOF): calcd for $C_{17}H_{35}N_2O_6Si^+$ ($[M+H]^+$) 391.2259, found 391.2287.

Synthesis of Gln-SNAc



Gln-SNAc was prepared by modifying a previously reported literature procedure for the synthesis of CoA-S-Glu.[5] **S1** (145 mg, 0.296 mmol, 1.0 equiv), N-acetylcysteamine (35 mg, 0.296 mmol, 1.0 equiv), EDC (108 mg, 0.592 mmol, 2.0 equiv) and HOAt (81 mg, 0.592 mmol, 2.0 equiv) were dissolved in 1.0 mL of DMF. Triethylamine (124 µL, 0.888 mmol, 3.0 equiv) was added dropwise and the reaction mixture was stirred overnight at room temperature. Following addition of sat. aq. $NaHCO_3$, the mixture was extracted with EtOAc twice and the combined organic extracts were washed with sat. aq. NaCl. Purification by flash column chromatography (7:3 hexanes:EtOAc to pure EtOAc) afforded protected Gln-SNAc (**S2**, 35 mg, 20% yield). Deprotection of **S2** was performed according to a previously reported literature procedure using a combination of TFA (10 equiv) and Et_3SiH (5 equiv) in DCM at rt, followed by dissolution in 0.1% v/v aq. TFA solution. After 5 h, the mixture was centrifuged (4,000 rpm, 10 min) and the supernatant was collected and concentrated. Purification by preparative HPLC (gradient: 100% H_2O to 90:5:5 H_2O:MeOH:MeCN over 13 min then hold 90:5:5 H_2O:MeOH:MeCN for 2 min) provided product that was deemed to be sufficiently pure by ^1H NMR and LCMS for subsequent enzymatic reaction with GloE and Q4Ox.

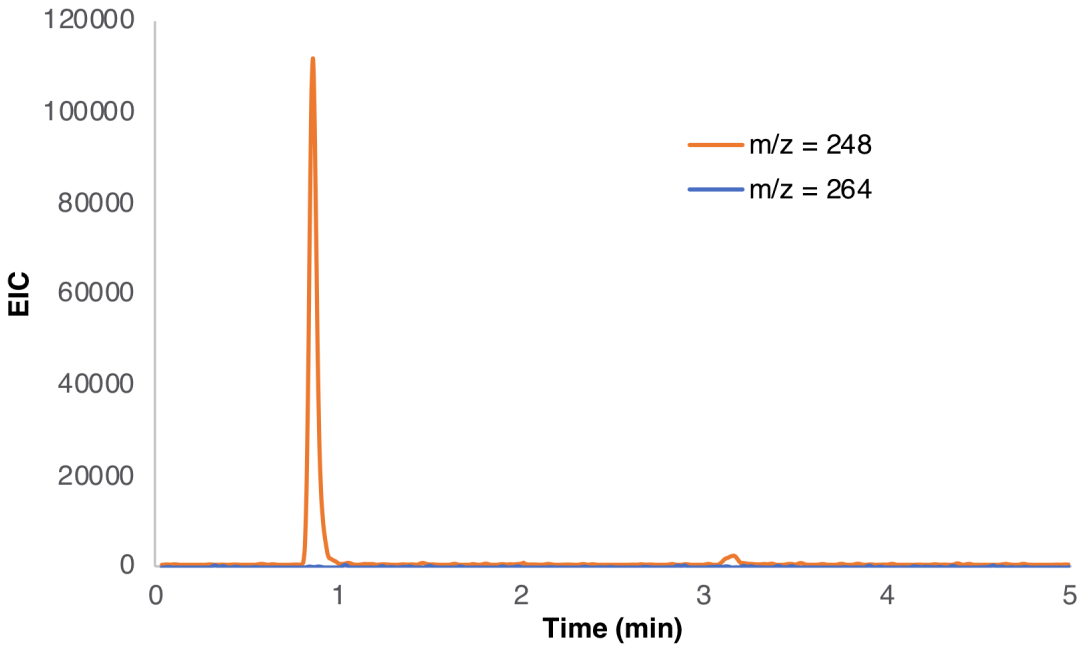
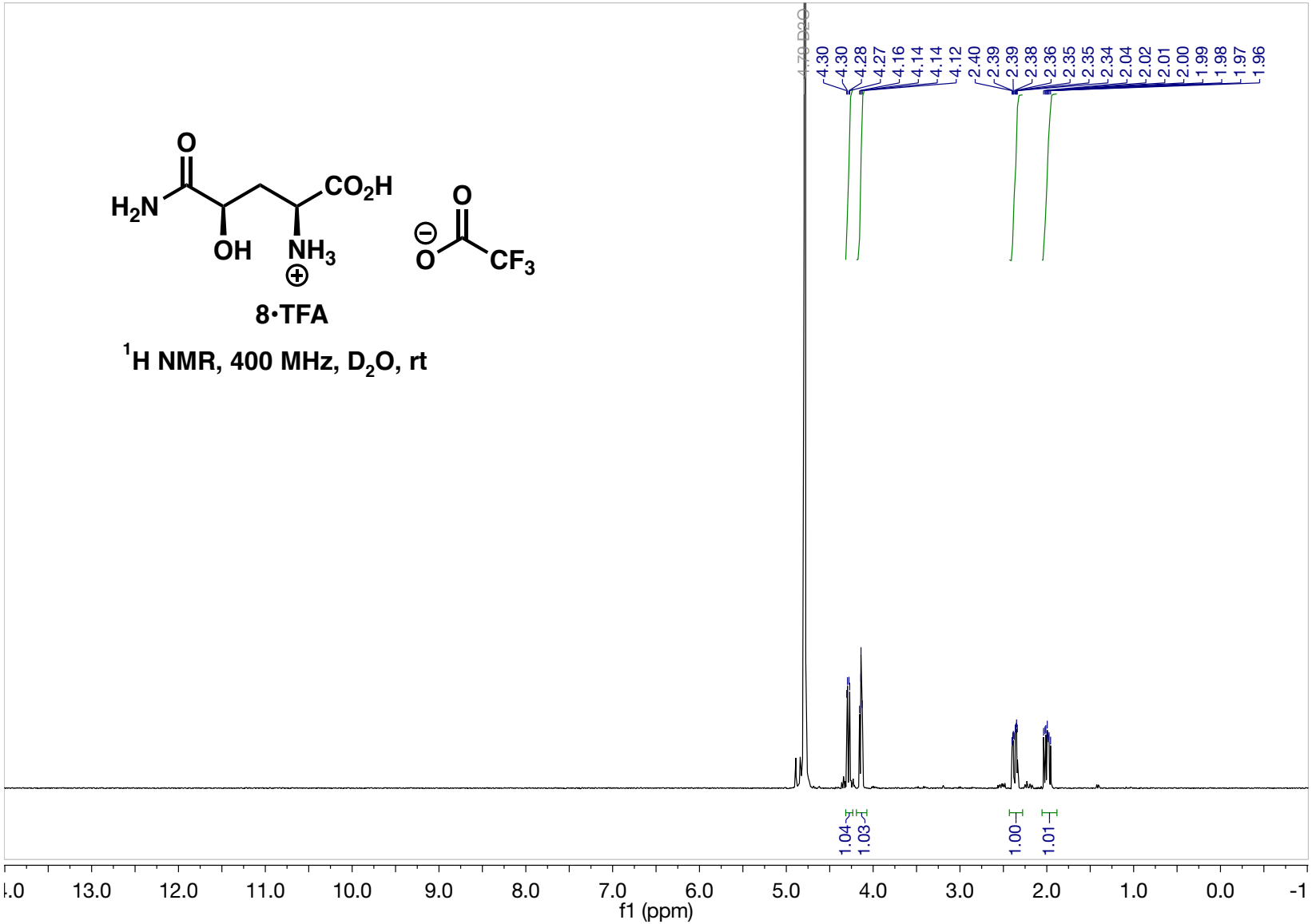
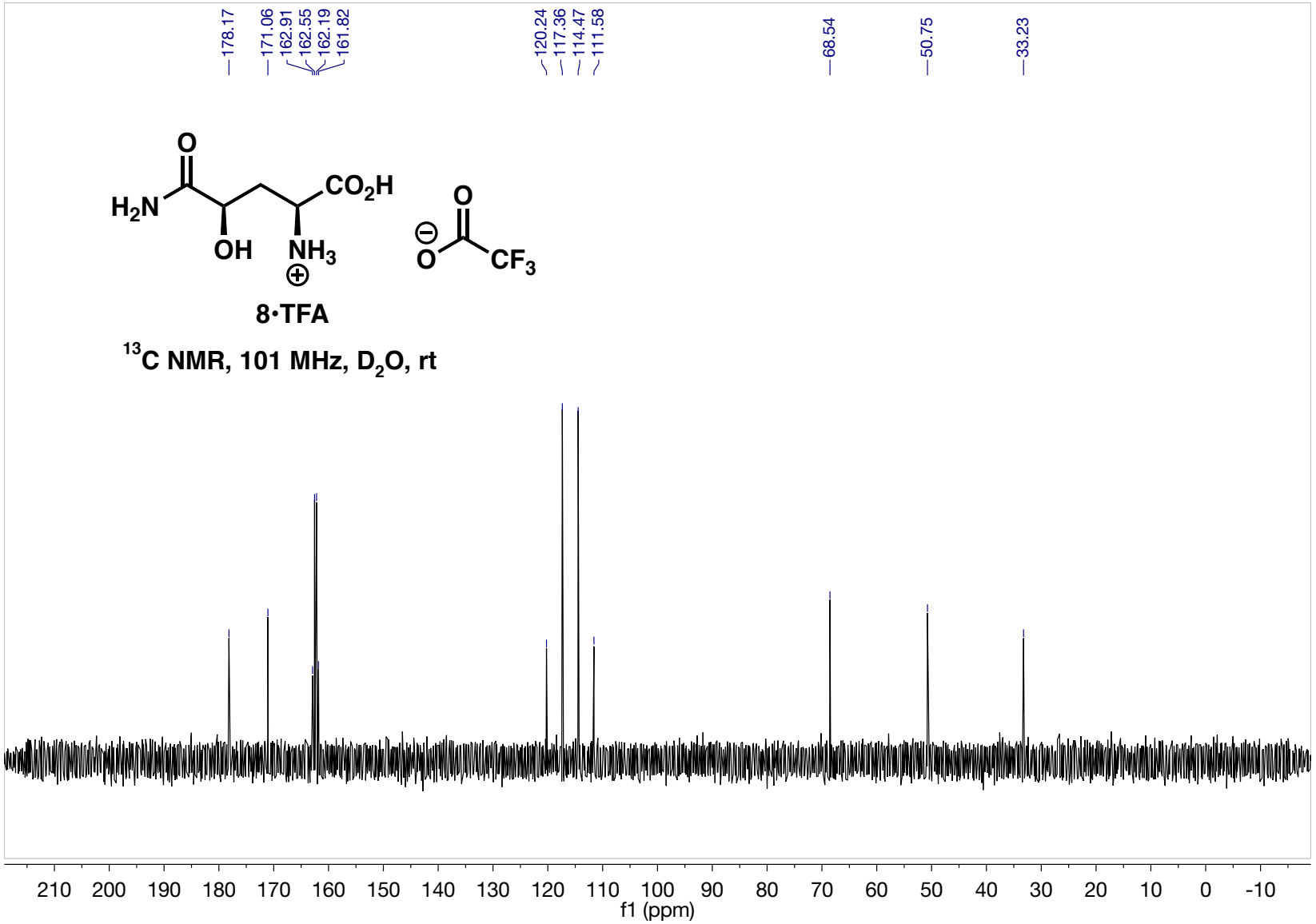


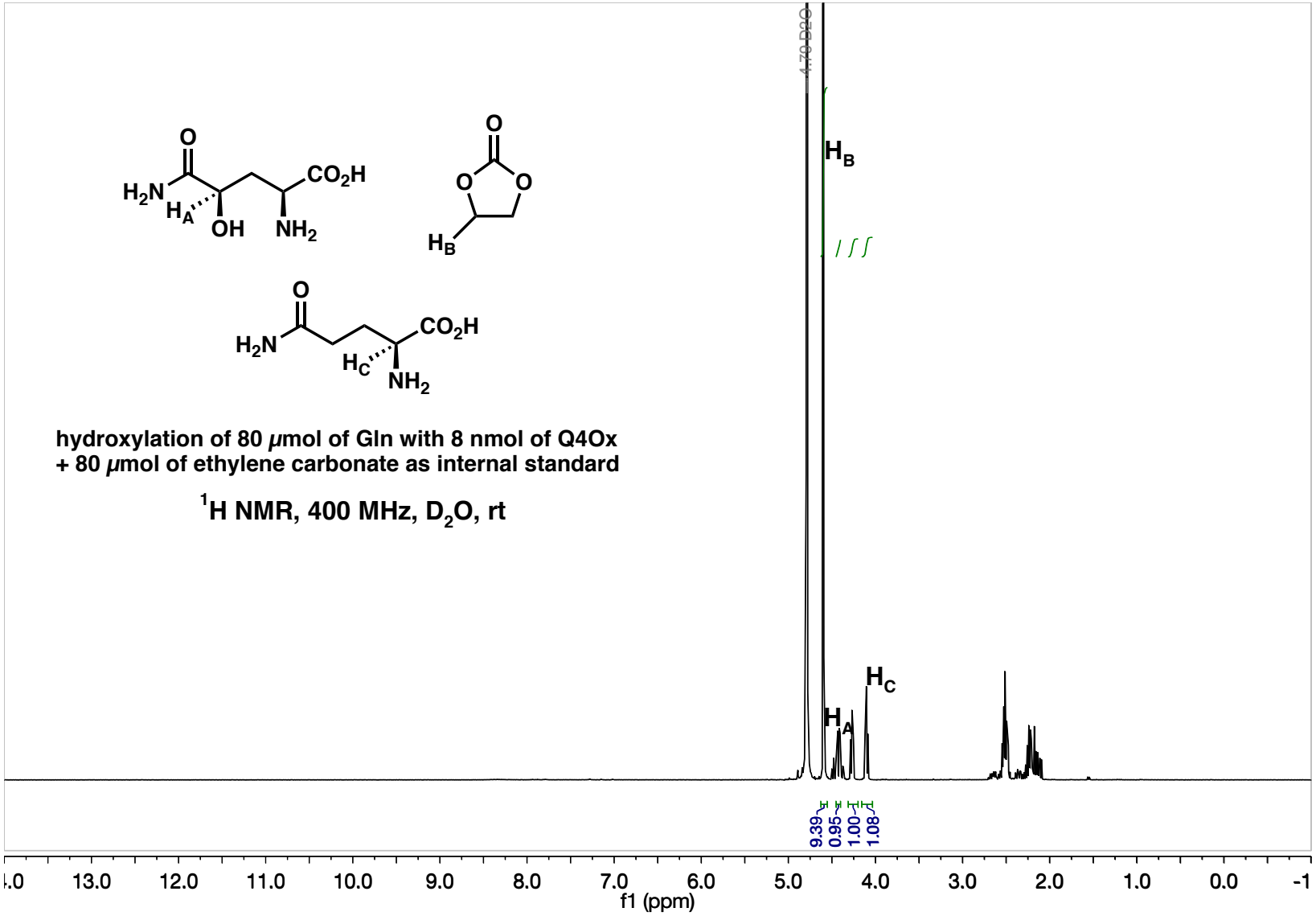
Figure S2. LCMS trace of Gln-SNAc hydroxylation with purified GloE. m/z (Gln-SNAc, $[M+H^+]$) = 248, m/z (3-OH-Gln-SNAc, $[M+H^+]$) = 264.

References

1. Cardani, S.; Prati, L.; Tinti, O. *Synthesis* **1986**, 1032–1035.
2. Ajish Kumar, K. S.; Chattopadhyay, S. *RSC Adv.* **2015**, *5*, 19455–19464.
3. Zwick, C. R., III; Sosa, M. B.; Renata, H. *J. Am. Chem. Soc.* **2021**, *143*, 1673–1679.
4. Prechter, A.; Heinrich, M. R. *Synthesis* **2011**, *10*, 1515–1525.
5. Strieker, M.; Nolan, E. M.; Walsh, C. T.; Marahiel, M. A. *J. Am. Chem. Soc.* **2009**, *131*, 13523–13530.

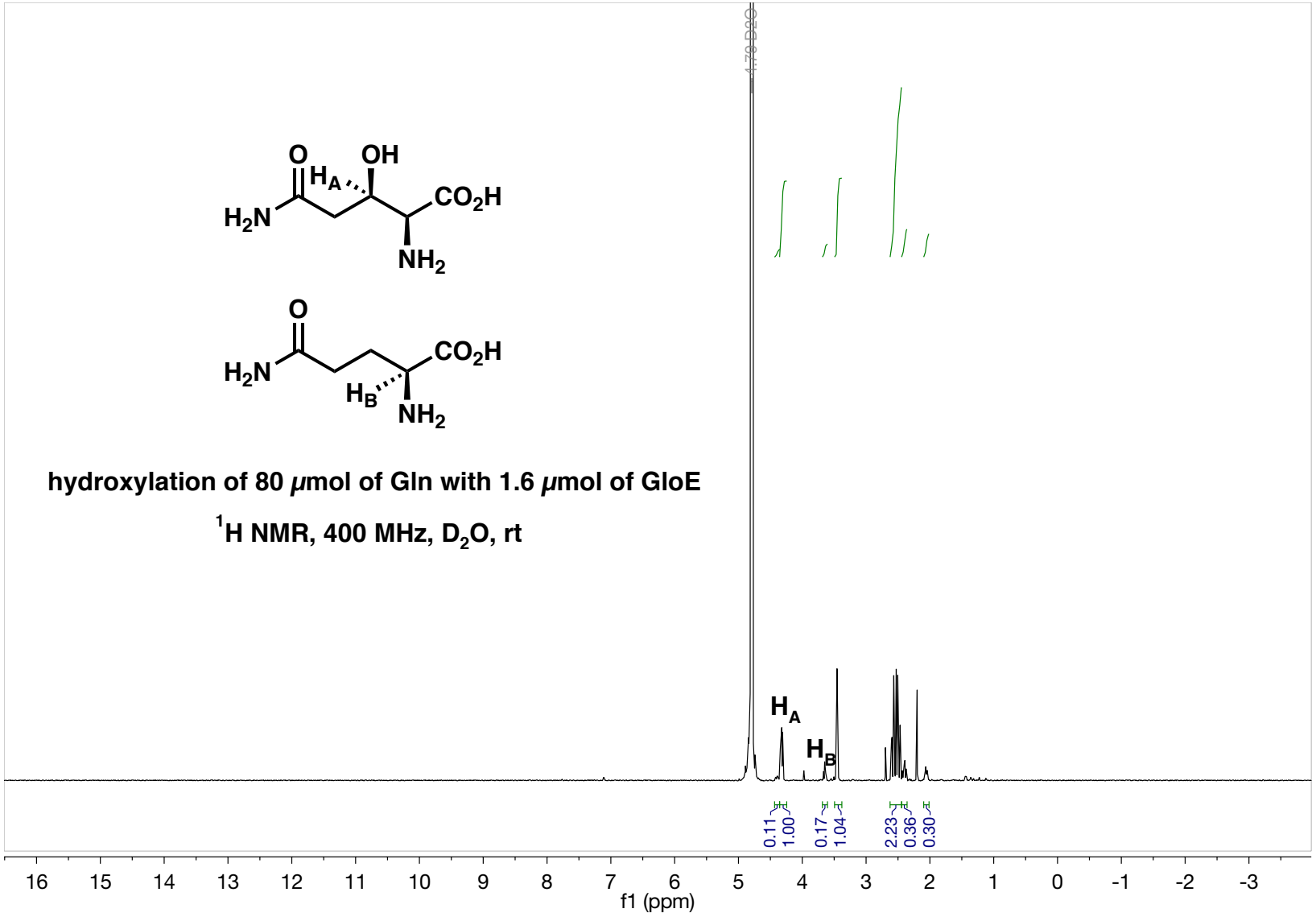

8·TFA
1H NMR, 400 MHz, D2O, rt
4.30
4.30
4.28
4.27
4.16
4.14
4.14
4.12
2.40
2.39
2.39
2.38
2.36
2.35
2.35
2.34
2.04
2.02
2.01
2.00
1.99
1.98
1.97
1.96
1.04
1.03
1.00
1.01
f1 (ppm)

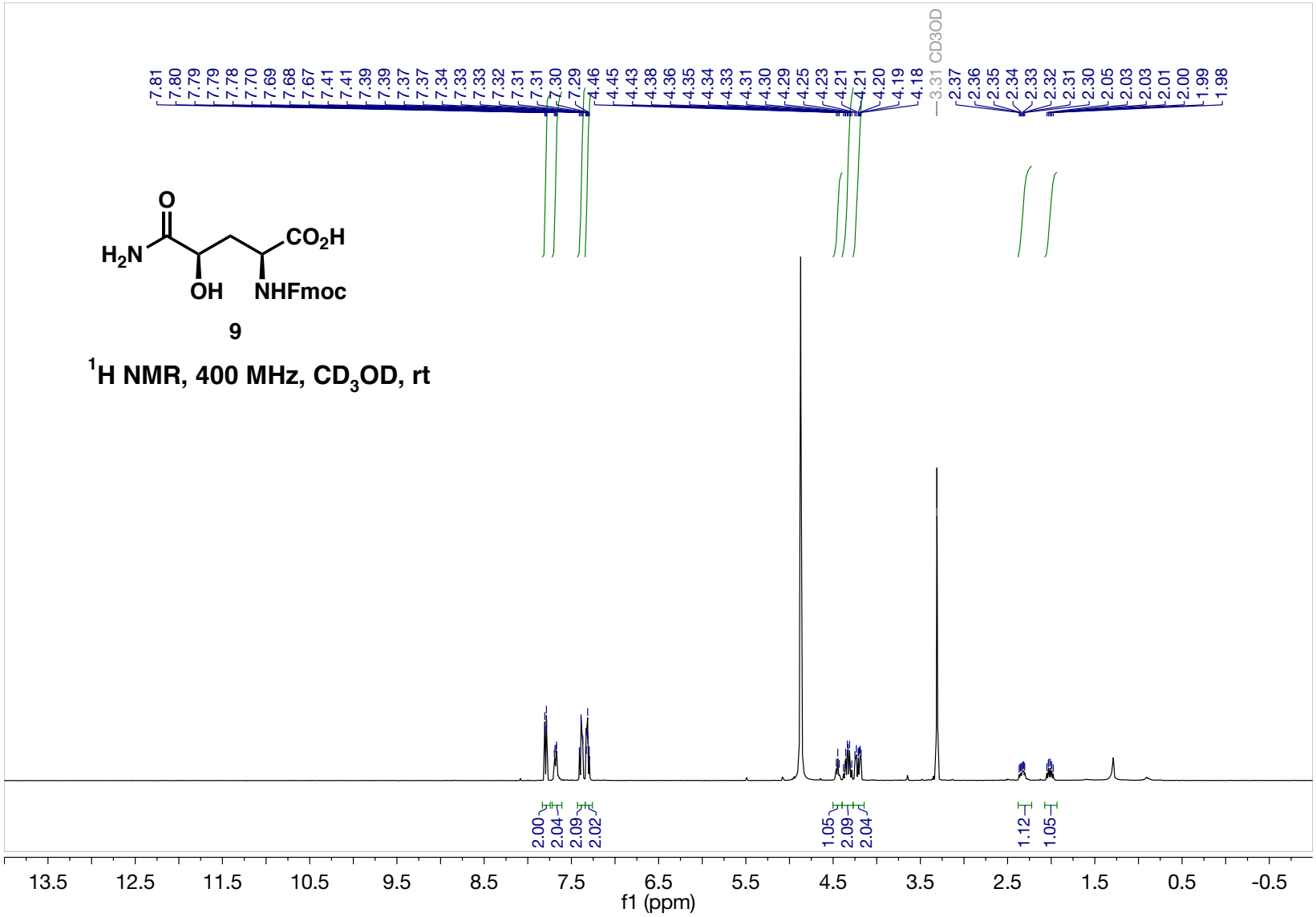

178.17
171.06
162.91
162.55
162.19
161.82
120.24
117.36
114.47
111.58
68.54
50.75
33.23
O
H2N
CO2H
OH
NH3
O
O
CF3
8·TFA
13C NMR, 101 MHz, D2O, rt
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)

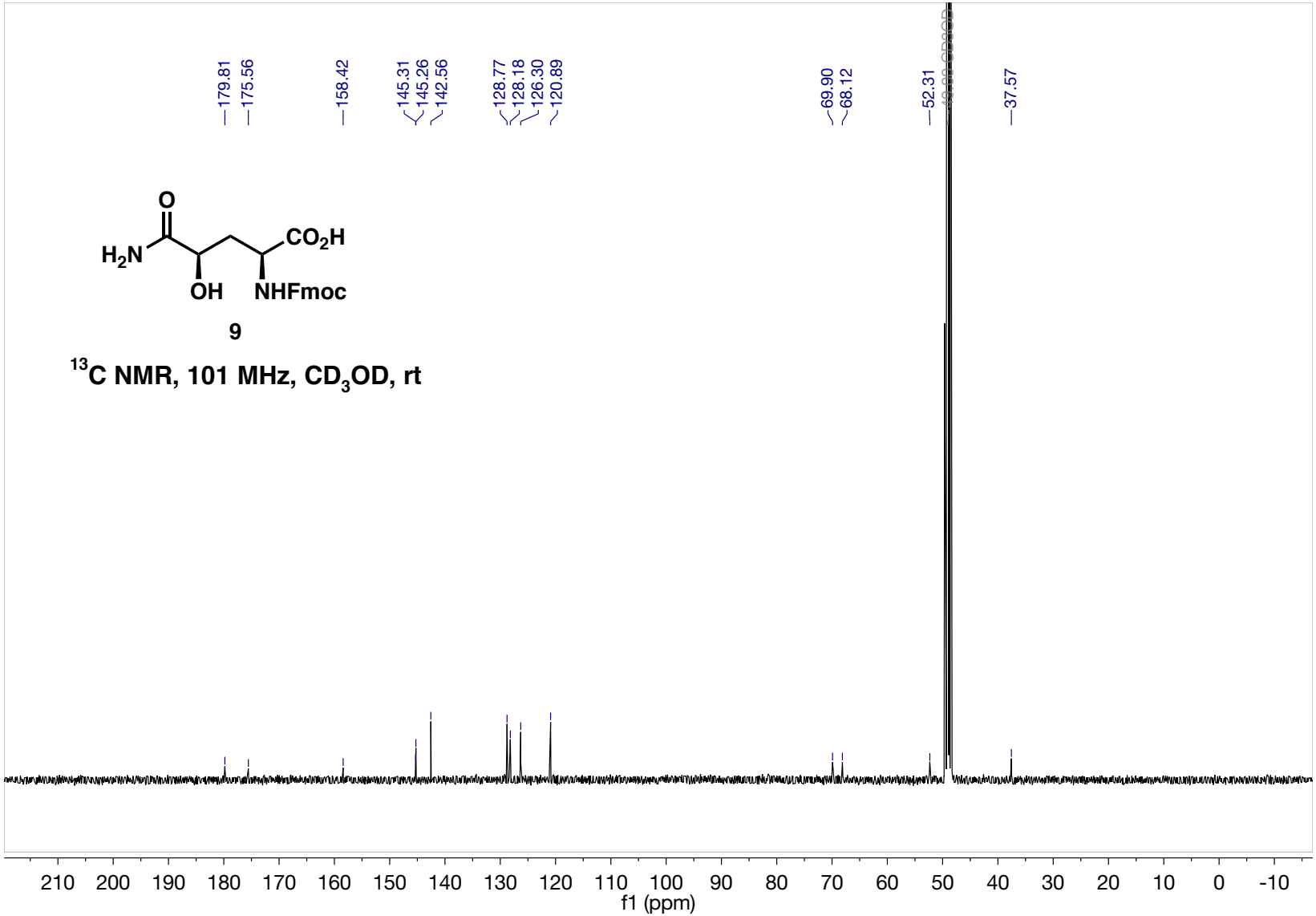


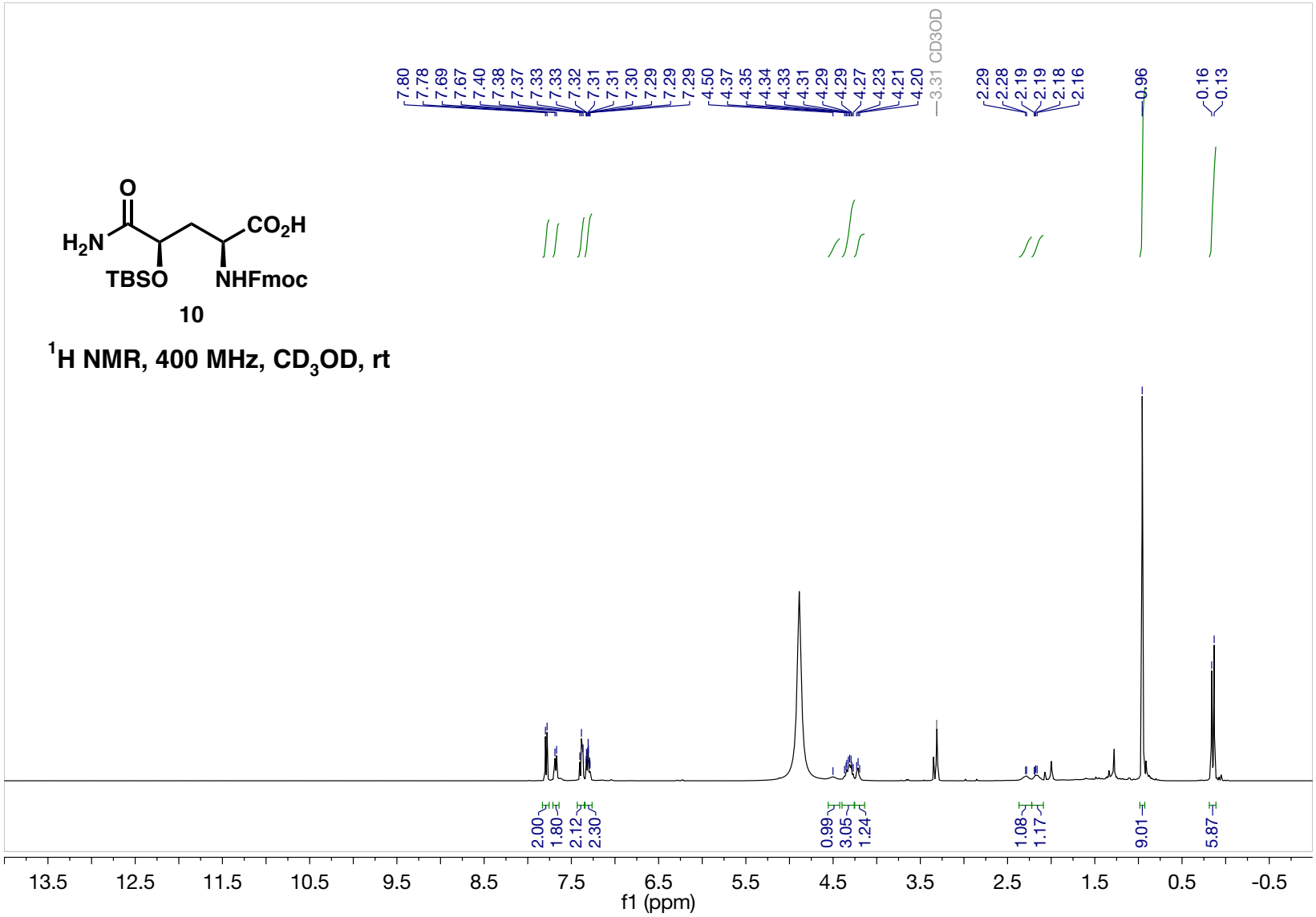
hydroxylation of 80 μmol of Gln with 8 nmol of Q4Ox + 80 μmol of ethylene carbonate as internal standard

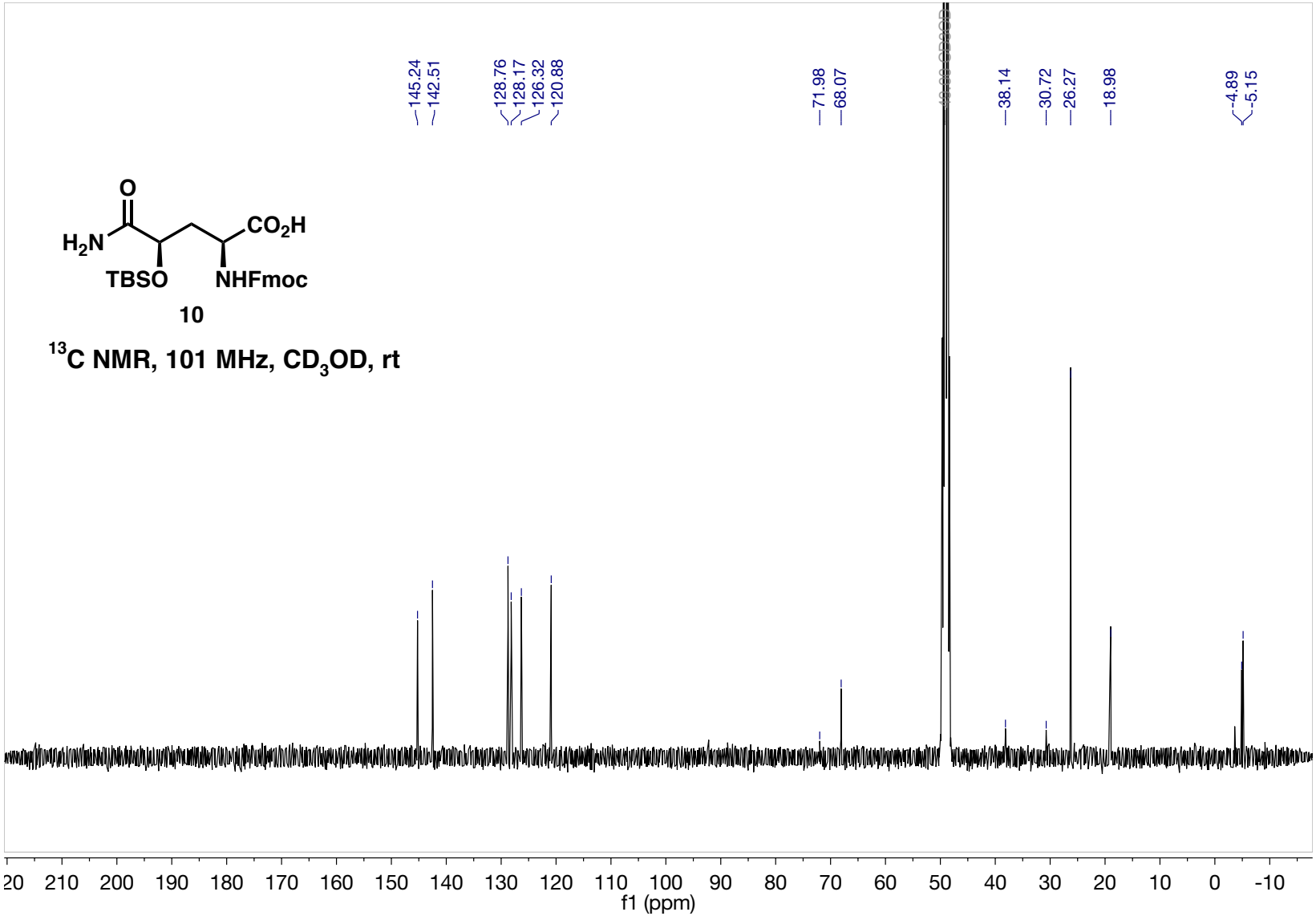
^{1}H NMR, 400 MHz, D_2O, rt

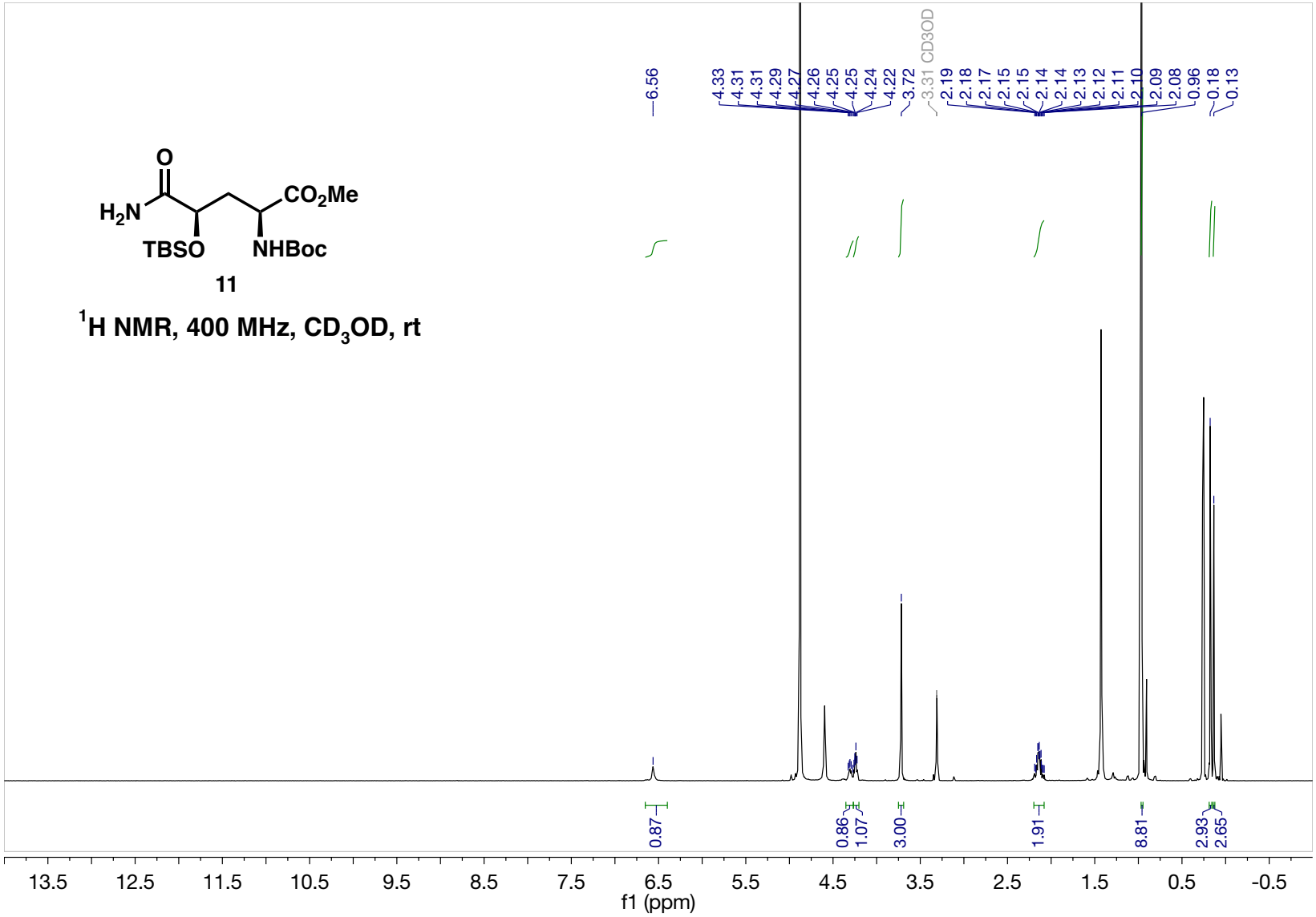

O
OH
H_A
H_2N
CO_2H
NH_2
O
H_2N
CO_2H
H_B
NH_2
hydroxylation of 80 μmol of Gln with 1.6 μmol of GloE
^{1}H NMR, 400 MHz, D_2O, rt
H_A
H_B
0.11
1.00
0.17
1.04
2.23
0.36
0.30
16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1 0 -1 -2 -3
f1 (ppm)

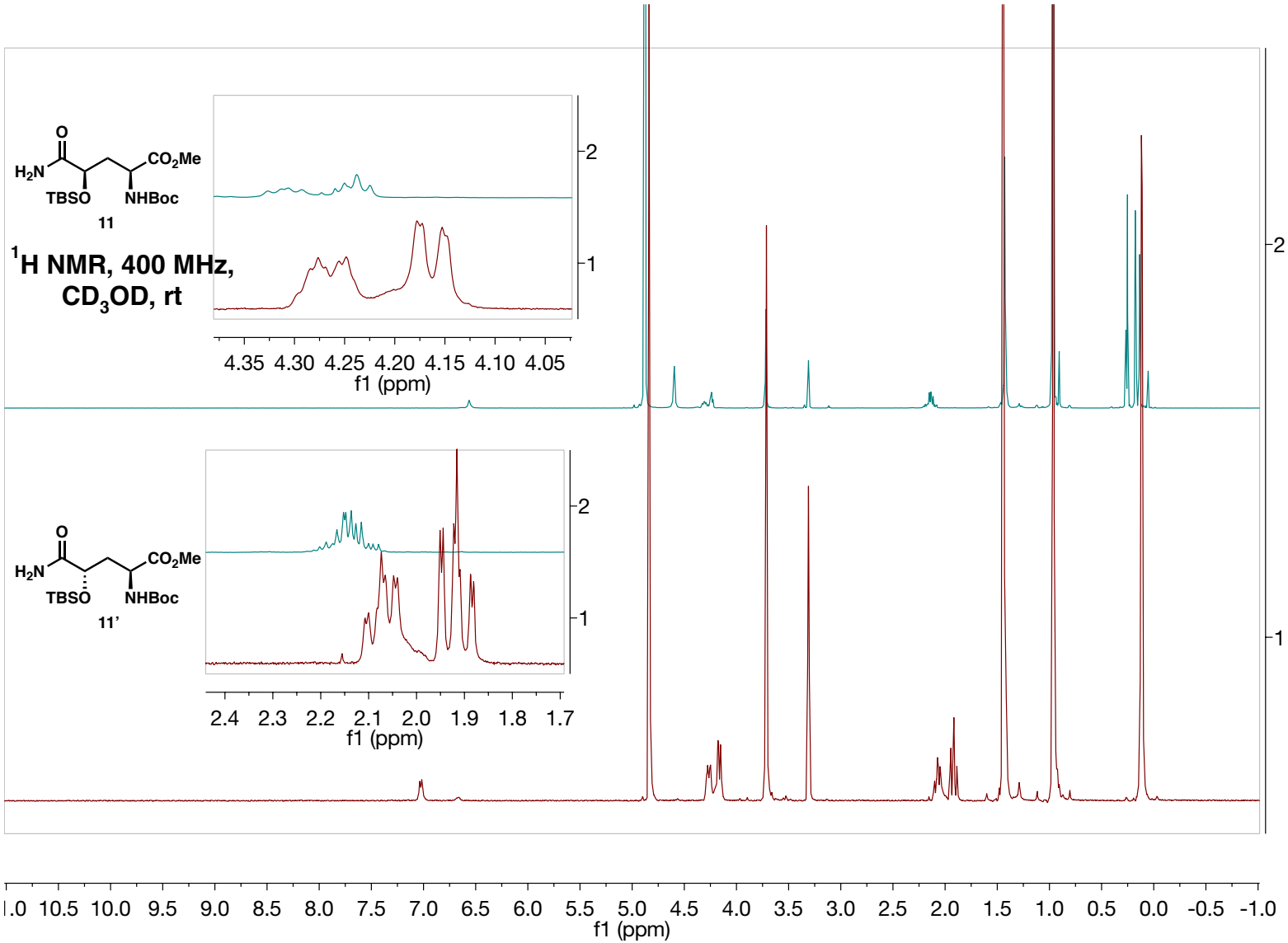

7.81 7.80 7.79 7.79 7.78 7.70 7.69 7.68 7.67 7.41 7.41 7.39 7.39 7.37 7.37 7.34 7.33 7.33 7.32 7.31 7.31 7.30 7.29 4.46 4.45 4.43 4.38 4.36 4.35 4.34 4.33 4.31 4.30 4.29 4.25 4.23 4.21 4.21 4.20 4.19 4.18 3.31 CD3OD 2.37 2.36 2.35 2.34 2.33 2.32 2.31 2.30 2.05 2.03 2.03 2.01 2.00 1.99 1.98
O
H2N
CO2H
OH
NHFmoc
9
1H NMR, 400 MHz, CD3OD, rt
2.00 2.04 2.09 2.02 1.05 2.09 2.04 1.12 1.05
13.5 12.5 11.5 10.5 9.5 8.5 7.5 6.5 5.5 4.5 3.5 2.5 1.5 0.5 -0.5
f1 (ppm)

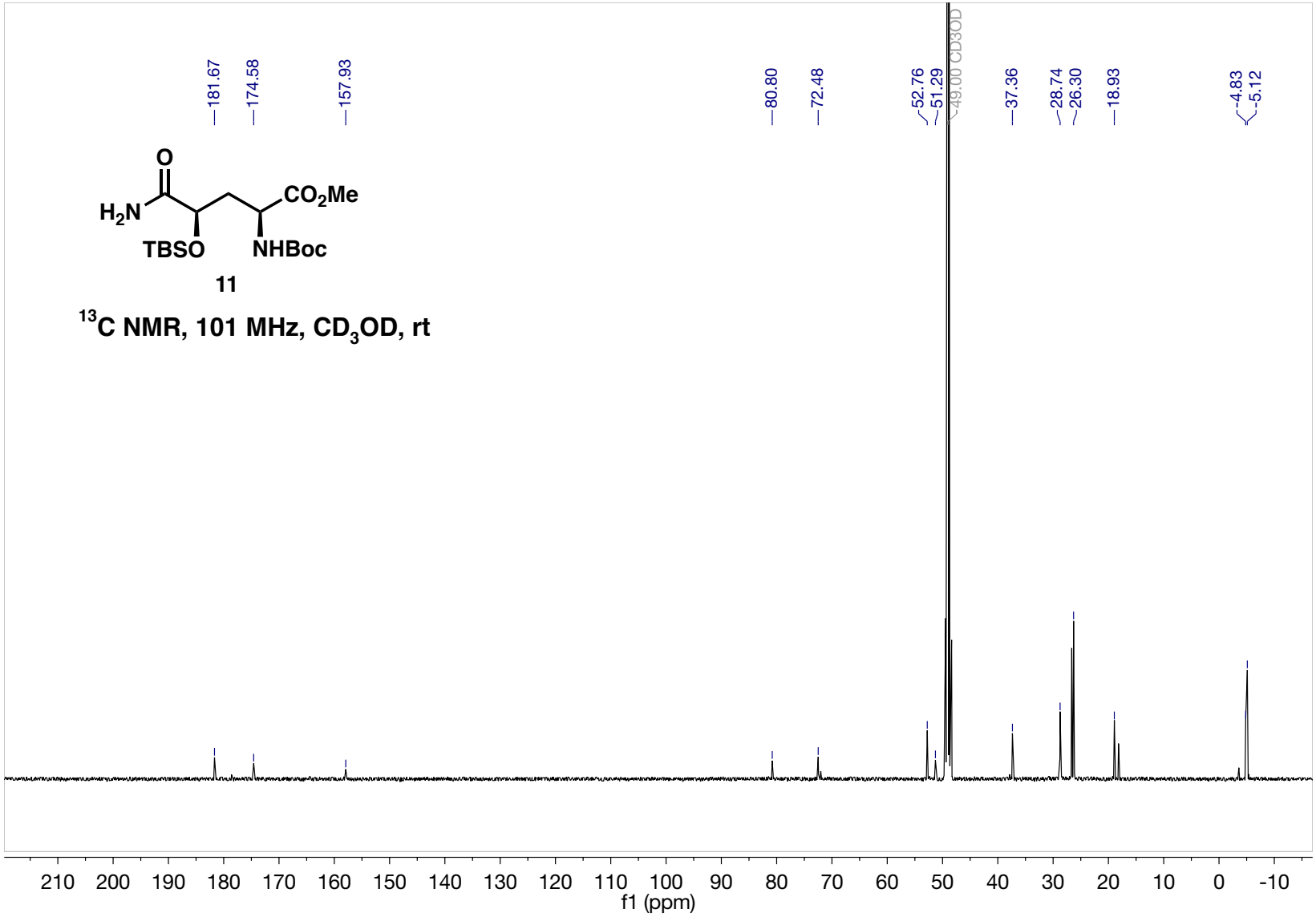

179.81
175.56
158.42
145.31
145.26
142.56
128.77
128.18
126.30
120.89
69.90
68.12
52.31
37.57
O
H2N
CO2H
OH
NHFmoc
9
13C NMR, 101 MHz, CD3OD, rt
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)


7.80
7.78
7.69
7.67
7.40
7.38
7.37
7.33
7.33
7.32
7.31
7.31
7.30
7.29
7.29
7.29
4.50
4.37
4.35
4.34
4.33
4.31
4.29
4.29
4.27
4.23
4.21
4.20
3.31 CD3OD
2.29
2.28
2.19
2.19
2.18
2.16
0.96
0.16
0.13
O
H2N
CO2H
TBSO
NHFmoc
10
1H NMR, 400 MHz, CD3OD, rt
2.00
1.80
2.12
2.30
0.99
3.05
1.24
1.08
1.17
9.01
5.87
13.5
12.5
11.5
10.5
9.5
8.5
7.5
6.5
5.5
4.5
3.5
2.5
1.5
0.5
-0.5
f1 (ppm)


145.24
142.51
128.76
128.17
126.32
120.88
71.98
68.07
49.00 CD3OD
38.14
30.72
26.27
18.98
-4.89
-5.15
O
H2N
CO2H
TBSO
NHFmoc
10
13C NMR, 101 MHz, CD3OD, rt
f1 (ppm)


O
H2N
CO2Me
TBSO
NHBoc
11
1H NMR, 400 MHz, CD3OD, rt
6.56
4.33
4.31
4.31
4.29
4.27
4.26
4.25
4.25
4.24
4.22
3.72
3.31 CD3OD
2.19
2.18
2.17
2.15
2.15
2.14
2.14
2.13
2.12
2.11
2.10
2.09
2.08
0.96
0.18
0.13
0.87
0.86
1.07
3.00
1.91
8.81
2.93
2.65
13.5
12.5
11.5
10.5
9.5
8.5
7.5
6.5
5.5
4.5
3.5
2.5
1.5
0.5
-0.5
f1 (ppm)


O
H2N
CO2Me
TBSO
NHBoc
11
1H NMR, 400 MHz,
CD3OD, rt
4.35 4.30 4.25 4.20 4.15 4.10 4.05
f1 (ppm)
11'
2.4 2.3 2.2 2.1 2.0 1.9 1.8 1.7
f1 (ppm)
1.0 10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0.5 -1.0
f1 (ppm)


181.67
174.58
157.93
80.80
72.48
52.76
51.29
49.00 CD3OD
37.36
28.74
26.30
18.93
-4.83
-5.12
O
H2N
CO2Me
TBSO
NHBoc
11
13C NMR, 101 MHz, CD3OD, rt
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)