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Supplementary Information

HAMA: A multiplexed LC-MS/MS assay for specificity profiling of adenylate-forming enzymes

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1 Synthesis of amino acid hydroxamates

1.1 General procedure

All reagents, amino acid methyl esters and solvents were obtained from commercial suppliers and used without further purification. Amino acid hydroxamates (XaaHAs) were synthesized by treating corresponding amino acid methyl esters with hydroxylamine according to previously published protocols.[1–7] Amino acid methyl ester hydrochloride (0.25-1 g) was dissolved in 10-15 mL MeOH and neutralized by careful dropwise addition of one equivalent of 0.6 M KOH in methanol while stirring on ice. The solution was filtered through a teflon 0.24 µm filter (Labsolute) to remove the precipitated KCl. A solution of hydroxylamine (1 M, 200 mL) was prepared freshly by mixing 140 mL of 1.43 M hydroxylamine hydrochloride solution in methanol with 60 mL of 3.33 M KOH solution in methanol with vigorous stirring on ice. After 30 min, solution was filtered to remove precipitated KCl. Calculated volume of neutralized hydroxylamine solution was added to the neutralized amino acid ester solution up to a final molar ratio of ester and hydroxylamine of 1:6. Reactions were stored at 4°C without stirring to facilitate crystallization. The formation of hydroxamates was detected by formation of a colored Fe^{3+} complex with 3% $FeCl_3$ in 0.1 M perchloric acid in ethanol. In general, hydroxamates of nonpolar amino acids crystallized spontaneously from the reaction mixture after 1-7 days, while polar ones required the addition of organic solvents. Precipitate was filtered, washed with dry methanol, dried under vacuum and stored at -20°C. Yields of hydroxamates were typically low (<20 %) due to the crystallization conditions which were not optimized.

Identity of hydroxamates was confirmed by high resolution mass spectrometry (HRMS; SI Table 3) and NMR (Section 11).

Hydroxamate	Synthetic procedure	NMR shifts of impurities
GlyHA[1,4]	Methyl ester was found to be prone to hydrolysis to free acid in alkaline hydroxylamine solution. Therefore, an incompletely neutralized hydroxylamine solution was used, which was prepared by mixing 13.9 g of hydroxylamine hydrochloride with 8.9 g KOH in 200 mL of methanol.	δ_H 4.02, s (presumably alpha proton of O-glycyl hydroxylamine, 20%)
AlaHA[1]	Reaction mixture concentrated to half the volume to facilitate crystallization. Crystallized after 2 days of storage at 4°C.	δ_H 3.29, s (methanol)
SerHA[4]	Reaction evaporated to dryness, dissolved in methanol and filtered to remove KCl. Diethylether (DET) was added with stirring until the solution turned cloudy. After 30 minutes of stirring, the solution cleared leaving resin on the flask wall. Resin was washed with DET, dried and stored at 4°C.	
ThrHA	Reaction evaporated to dryness and redissolved in a small amount of methanol. Methyl tert-butyl ether (TBME) was added while stirring until the solution turned cloudy. After 15 min of stirring,	δ_H 3.32, s (methanol) δ_H 1.19, s; 3.20, s (MTBE) δ_C 27.4; 50.4 (MTBE)

	ThrHA precipitated as a white, hygroscopic solid which was carefully filtered, washed with TBME and dried.	
Cys_2HA	Cys methyl ester was found to be unstable in alkaline reaction conditions. Therefore, cystine methyl ester was employed for the synthesis of the hydroxamate. Cystine methyl ester dihydrochloride was neutralized with 2 equivalents of KOH and treated with 12 equivalents of hydroxylamine. After two days, reaction was concentrated to half volume and left to precipitate at 4°C overnight. Cysteine hydroxamate is prepared by reducing cystine hydroxamate with 3 equivalents of tris(2-carboxyethyl)phosphine hydrochloride in water at 60°C for 10 min.	δ_H 3.89, s (amino acid methyl ester)
ValHA[1]	Precipitated from the reaction mixture after 3 days of storage at 4°C.	
LeuHA[5]	Precipitated from the reaction mixture after 7 days of storage at 4°C.	δ_H 4.01, dd (alpha proton of free amino acid, 10%)
IleHA[1,5]	Precipitated from the reaction mixture after 7 days of storage at 4°C.	δ_H 3.98, d (alpha proton of free amino acid, 20%) δ_C 172.9 (alpha carbon of free amino acid)
MetHA[5]	Precipitated from the reaction mixture after 2 days of storage at 4°C.	δ_H 4.20, dd (alpha proton of free amino acid, 6%) δ_C 173.0 (alpha carbon of free amino acid)
ProHA*HCl	Reaction evaporated to dryness and redissolved in a small amount of methanol while heating to 60°C. Solution acidified with concentrated HCl under vigorous stirring. DET was added to the solution until it turned cloudy. After 30 min of stirring at room temperature, the hydroxamate precipitated as translucent resin on the flask wall. Solvent was decanted, resin washed with DET, dissolved in methanol, filtered to remove KCl and dried.	δ_H 1.13, d (isopropanol) δ_H 1.89, s (ethyl acetate) δ_H 3.32, s (methanol) δ_H 4.42, dd (alpha proton of free amino acid, 12%)
PheHA[1,2,6,7]	Precipitated from the reaction mixture after 2 days of storage at 4°C.	δ_H 2.50, s (DMSO) δ_C 39.5 (DMSO)
TyrHA[6,7]	Methyl ester was provided as a free base, so the KOH neutralization step was omitted and the ester dissolved directly in hydroxylamine solution. Precipitated from the reaction mixture after 2 days of storage at 4°C.	δ_H 3.30, s (methanol) δ_C 50.3 (methanol)
TrpHA[6]	Reaction evaporated to dryness, redissolved in isopropanol and heated to 60°C until a rose-white	δ_H 1.10, d; 3.96, sept (isopropanol)

	precipitate formed which was filtered, washed with isopropanol and dried.	δ_H 4.35, dd (alpha proton of free amino acid, 9%) δ_C 173.2 (alpha carbon of free amino acid)
AspHA	Methyl ester provided as a free base, so the KOH neutralization step was omitted. Ester was dissolved directly in methanolic hydroxylamine solution. Asp methyl ester was found to be prone to hydrolysis to free acid in alkaline hydroxylamine solution. Therefore, an incompletely neutralized hydroxylamine solution was used, which was prepared by mixing 13.9 g of hydroxylamine hydrochloride with 8.9 g KOH in 200 mL of methanol.	δ_H 3.85, s (amino acid methyl ester) δ_H 4.40, dd (alpha proton of free amino acid, 8%) δ_H 4.47, dd (alpha proton of amino acid methyl ester, 3%)
GluHA	Methyl ester was provided as a free base, so the KOH neutralization step was omitted. Ester was dissolved directly in methanolic hydroxylamine solution. Water added dropwise until complete dissolution and the reaction mixture stored at 4°C.	δ_H 3.34, s (methanol) δ_H 4.40, dd (alpha proton of free amino acid, 6%)
HisHA[6]	Reaction evaporated to dryness and redissolved in a small amount of methanol. Isopropanol was added to the solution until a white, extremely hygroscopic precipitate formed which was filtered, washed with isopropanol and dried.	δ_H 1.17, d; 4.02, sept (isopropanol) δ_H 3.35, s (methanol) δ_H 4.40, dd (alpha proton of free amino acid, 7%) δ_C 25.1; 65.6 (isopropanol)
LysHA	Precipitated from the reaction mixture after 2 days of storage at 4°C.	δ_H 4.04, dd (alpha proton of O-lysyl hydroxylamine, 5%) δ_H 4.48, dd (alpha proton of free amino acid, 7%)
ArgHA*2HCl	Reaction evaporated to dryness and redissolved in a small amount of methanol. Solution acidified with concentrated HCl with vigorous stirring. Isopropanol was added to the solution until a white, extremely hygroscopic precipitate formed which was filtered, washed with isopropanol and dried.	δ_H 1.13, d; 3.98, sept (isopropanol) δ_H 3.32, s (methanol)
PipHA	Reaction evaporated to dryness, dissolved in methanol and filtered to remove KCl, DET was added with stirring until the solution turned cloudy. After 30 minutes of stirring, the solution cleared leaving resin on the flask wall. Resin was washed with DET, dried and stored at 4°C.	δ_H 3.33, s (methanol)
Phenylglycine-HA[2]	Precipitated from the reaction mixture after 2 days of storage at 4°C.	δ_H 5.02, dd (alpha proton of O-phenylglycyl hydroxylamine, 3%) δ_H 5.42, dd (alpha proton of free amino acid, 11%)

β-PheHA	Reaction evaporated to dryness and redissolved in a small amount of methanol while heating to 60°C. Solution acidified with concentrated HCl under vigorous stirring. Diethylether (DET) was added to the solution until it turned cloudy. After 30 min of stirring at room temperature, the hydroxamate precipitated as translucent resin on the flask wall. Solvent was decanted, resin washed with DET, dissolved in methanol, filtered to remove KCl and dried.	δ_H 3.19, s (methanol)

1.2 Preparation and storage of standard solutions

Individual hydroxamates are stored as 10 mM solutions in 20 mM HCl at -20°C. Very hygroscopic compounds (ArgHA, HisHA, ProHA, SerHA, ThrHA, PipHA, β-PheHA) are stored as 50 mM solutions in water at -20°C. The quantitation standard of amino acid hydroxamates is stored as acidic solution in water: 0.3 mM hydroxamates, 10 mM tris-(2-carboxyethyl) phosphine hydrochloride (TCEP) at -20°C. On the day of the analysis, the standard solution is diluted to 100 µM final concentration in 50 mM TRIS (pH 7.5), 150 mM hydroxylamine (pH 7.5-8), 5 mM ATP. This solution is diluted with the buffer containing assay components (50 mM TRIS [pH 7.5], 150 mM hydroxylamine [pH 7.5-8], 5 mM ATP) to obtain standard solutions (0.032-100 µM) mimicking the assay conditions. All standards are further diluted 10-fold in 95% acetonitrile + 0.1% formic acid before UPLC-MS/MS analysis (0.0032-10 µM). Diluted hydroxamate standards are freshly prepared and used in the course of one day.

1.3 UPLC-ESI-HRMS analysis of amino acid hydroxamates

Exact masses of the synthetic hydroxamates were confirmed by high resolution mass spectrometry (**SI Table 3**) on a Dionex Ultimate3000 system combined with a Q-Exactive Plus mass spectrometer (Thermo Scientific) with a heated electrospray ion source (HESI). All masses were detected by ESI as $M+H^+$ adducts in positive mode. The measurement was carried out within a mass range of m/z 50 – 400.

2 Enzymatic product formation under competition is governed by the specificity constant k_{cat}/K_M

The relative product formation rates for two substrates can be derived under steady state conditions in analogy to the Michaelis-Menten equation.[8] The product is formed from the corresponding Michaelis complexes in an irreversible, monomolecular reaction (Eq. 1.1 and 1.2). Under the assumption that the concentrations of both Michaelis complexes remain constant, their concentration can be expressed as a function of the Michaelis constants (e.g. $K_{M1} = [k_{-1} + k_2]/k_1$) and the substrate concentrations (Eq. 2.1 and 2.2). Inserting Eq. (2) into Eq. (1) results in Eq. (3) which describes the ratio of product formation rates which are proportional to the corresponding specificity constants k_2/K_{M1} and k_4/K_{M2} multiplied with the respective substrate concentrations.

$$E + A_1 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EA_1 \overset{k_2}{\rightarrow} E + P_1$$

$$E + A_2 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EA_2 \overset{k_4}{\rightarrow} E + P_2$$

$$\frac{d[P_1]}{dt} = k_2[EA_1] = v_1 \qquad (1.1)$$

$$\frac{d[P_2]}{dt} = k_4[EA_2] = v_2 \qquad (1.2)$$

$$[EA_1] = \frac{K_{M2}[E]_0[A_1]}{K_{M2}[A_1]+K_{M1}[A_2]+K_{M1}K_{M2}} \qquad (2.1)$$

$$[EA_2] = \frac{K_{M1}[E]_0[A_2]}{K_{M2}[A_1]+K_{M1}[A_2]+K_{M1}K_{M2}} \qquad (2.2)$$

$$\frac{v_1}{v_2} = \frac{\frac{k_2}{K_{M1}}[A_1]}{\frac{k_4}{K_{M2}}[A_2]} \qquad (3)$$

3 Cloning

3.1 General cloning

General cloning was carried out in *E. coli* strain NEB 5-alpha (New England Biolabs). Protein expression was carried out in *E. coli* strains NEB BL21 or HM0079.[9] Preparation of plasmid DNA, gel purification of DNA fragments, and purification of PCR products were performed using NucleoSpin Plasmid and Gel and PCR clean-up kits (Macherey Nagel). Purification of the genomic DNA was performed according to a published protocol.[10] PCRs were carried out with Q5 polymerase (New England Biolabs, Massachusetts) or Phusion High-Fidelity DNA Polymerase (New England Biolabs), according to the supplier's instructions. PCR fragments carrying vector-specific overhangs were cloned into vectors linearized by restriction digestions using the InFusion cloning kit (Takara Bio Europe). Oligonucleotide primers (Section 3.3) were made by custom synthesis and sequence confirmation of PCR amplified inserts was performed using the Mix2Seq service for Sanger sequencing (Eurofins Genomics).

3.2 Plasmids

pSU18 and pTrc99a vectors[9] were linearized with NcoI and BamHI while pOPINE[11] was linearized with NcoI and PmeI restriction enzymes. pSU18-TycA, pSU18-sdVGrsA,[12] pMG211-Sfp[13] and pTrc99a-GrsB_MtoL[14] plasmids were kindly provided by Prof. Donald Hilvert (ETH Zurich). The gene encoding the A-T didomain of the Jes-A1 module was amplified from *Pseudomonas aeruginosa* QS1027 genomic DNA[15] and cloned into pTrc99a. Genes encoding the SrfA-C, SrfA-A1 and SrfA-B2 modules were amplified as C-A-T constructs (SrfA-C as C-A-T-Te) by PCR from *Bacillus subtilis* 3610 genomic DNA and cloned into pTrc99a. The genes encoding all four GrsB modules were amplified as C-A-T constructs (GrsB4 as C-A-T-Te) from pTrc99a-GrsB_MtoL. *grsB1* was subcloned into pTrc99a, while *grsB2*, *grsB3* and *grsB4* were subcloned into pSU18. Aminoacyl-tRNA synthetase genes (*hisS*, *leuS*, *metG*) were amplified from *E. coli* NEB 5-alpha genomic DNA and cloned into the pOPINE vector.

To generate mutants of sdVGrsA for the directed evolution experiment, two fragments of *sdVgrsA* were amplified from pSU18-sdVGrsA using mutagenic primers and cloned into pSU18-sdVGrsA linearized with AflII and SacI. The first fragment was amplified with primer sdXGrsA_f and a suitable reverse primer. The second fragment was amplified with a mutagenic forward primer, e.g. D306S_f, and sdXGrsA_r.

3.3 Oligonucleotides used as primers

Overhangs for InFusion cloning are underlined.

SrfA-A1_f	CAA TTT CAC ACA GGA AAC AGA CCA TGT TAA CGG ATG CAC AAA AAC GA
SrfA-A1_r	TGG TGA TGG TGA TGA GAT CTG GAT CCT TCC TCT GCA AGA GCC GTA ATC
SrfA-B2_f	CAA TTT CAC ACA GGA AAC AGA CCA TGA AGG AGG AGC AGA CGT TTG AA
SrfA-B2_r	TGG TGA TGG TGA TGA GAT CTG GAT CCA GCA GAC GCC TCC ATA TAA GC
JesA1_f	CAA TTT CAC ACA GGA AAC AGA CCA TGC TCA ATG CCA GCG AAA CCG CG
JesA1_r	GGT GAT GGT GAT GAG ATC TGG ATC CAA TCT CGC CGC CCT TGC CAC
GrsB1_f	ATT TCA CAC AGG AAA CAG ACC ATG AGT ACA TTT AAA AAA GAA CAT GTT CAG G
GrsB1_r	TGG TGA TGA GAT CTG GAT CCC CCG TTT ATA TAA TTA GAG ATT TCC TGA ATG G
HisS_f	AGG AGA TAT ACC ATG GCA AAA AAC ATT CAA GCC A
HisS_r	GTG ATG GTG ATG TTT ACC CAG TAA CGT GCG CA
MetG_f	AGG AGA TAT ACC ATG ACT CAA GTC GCG AAG AAA ATT C

MetG_r	GTG ATG GTG ATG TTT TTT CAC CTG ATG ACC CGG T
LeuS_f	AGG AGA TAT ACC ATG CAA GAG CAA TAC CGC C
LeuS_r	GTG ATG GTG ATG TTT GCC AAC GAC CAG ATT GAG G
GrsB2_f	CAA TTA AGG AGG CAG CAG ATG ATT CAG CCT GTA CCA GAA CAA
GrsB2_r	GTG ATG GTG ATG AGA TCT GGA TCC ATC AGC AAT GTA TTG AGC TAA TG
SrfA-C_f	ATT TCA CAC AGG AAA CTC GAG ATG AGT CAA TTT AGC AAG GAT CAG G
SrfA-C_r	TGG TGA TGA GAT CTG GAT CCT GAA ACC GTT ACG GTT TGT GTA TTA AG
GrsB3_f	CAA TTA AGG AGG CAG CAG ATG ATT CAA CCT GTT ACC CCG
GrsB3_r	GTG ATG GTG ATG AGA TCT GGA TCC CTC CTC TAT ATA TTT AGC CAG TCC
GrsB4_f	CAA TTA AGG AGG CAG CAG ATG GCT ATT CAG CCG GT
GrsB4_r	CTT AGT GAT GGT GAT GGT GA

Primers for mutagenesis of sdVGrsA:

sdXgrsA_f	GAG CAT AAA GGA ATA AGT AAT CTT AAG G
D306S_f	CTT CGC TCC CTA ATT GTA GGT GGA AGC GCC TTG TCT CCG AAA CAC ATC
G243M_f	CGT ATA ATA CAG ACC GGA GCA ATT GGA TTC GAT GCA CTG ACA TTT GAA GTT TTT ATG TCA TTG CTG CAT GGA GCT GAA TTG
N334T_f	GAA CGG TTA CGG CCC AAC AGA AAC CAC CAC TTT TTC TAC ATG CTT TCT TAT TGA TAA AG
N334T_S338A_f	GAA CGG TTA CGG CCC AAC AGA AAC CAC CAC TTT TGC GAC ATG CTT TCT TAT TGA TAA AGA ATA TGA TGA CAA TAT TC
S338A_f	GAA CGG TTA CGG CCC AAC AGA AAA CAC CAC TTT TGC GAC ATG CTT TCT TAT TGA TAA AGA ATA TGA TGA CAA TAT TC
A356P_f	CTT TCT TAT TGA TAA AGA ATA TGA TGA CAA TAT TCC GAT AGG GAA GCC GAT TCA AAA TAC ACA AAT TTA TAT TGT CGA TGA TGA AAA TCT TC
D306_r	CCA CCT ACA ATT AGG GAG CGA AGG C
G243_r	GCT CCG GTC TGT ATT ATA CGA TCG
N334_r	CTG TTG GGC CGT AAC CGT TCC
A356_r	GTC ATC ATA TTC TTT ATC AAT AAG AAA GCA TGT AG
sdXgrsA_r	GCT AAC CCT TCT CCA CCA ATA CAG

4 Protein overexpression and purification

4.1 Purification protocol

For the overexpression of C-terminally His_6-tagged holo-NRPS proteins, each overexpression plasmid was transformed into *E. coli* HM0079 with genomically integrated 4'-phosphopantheteinyl transferase Sfp.[9] Overexpression of apo-TycA, Sfp and aminoacyl tRNA synthetases was done in *E. coli* BL21 strain. A 2 L flask with 500 mL of 2xYT medium supplemented with antibiotics was inoculated with 0.5 mL of an overnight culture and incubated at 37°C in a rotary shaker at 200 rpm. When the OD_{600} reached 1, cultures were induced with 0.25 mM isopropyl-D-thiogalactoside (IPTG) and grown for another 16-20 hours at 20°C. Cells were harvested by centrifugation and the supernatant was discarded. After resuspending the cell pellet in 30 mL lysis buffer (50 mM TRIS [pH 7.4], 500 mM NaCl, 20 mM imidazole, 2 mM TCEP), 100 µL protease inhibitor mix (Sigma, P8849) were added and cells were lysed by sonication while cooling on ice. The lysate was cleared by centrifugation at 19,000 g for 30 min at 4°C and the supernatant was loaded onto a column packed with 2 mL of Ni-IDA suspension (Rotigarose, Roth) and equilibrated with lysis buffer. After washing the column twice with 20 mL of the lysis buffer, the target protein was eluted with 4 x 0.75 mL elution buffer (50 mM TRIS [pH 7.4], 500 mM NaCl, 300 mM imidazole, 2 mM TCEP). After pooling the protein-containing fractions, they were buffer exchanged with 2-fold concentrated adenylation assay buffer (100 mM TRIS [pH 7.6], 10 mM $MgCl_2$) on 6 mL Vivaspin (Sartorius) filters with 10 kDa cut-off for proteins larger than 30 kDa and 30 kDa cut-off for proteins larger than 90 kDa. Glycerol was added to 10% and protein concentration adjusted to 50 µM. Samples were flash frozen in liquid nitrogen and stored at -20°C. For detailed kinetic analysis, TycA protein samples were further purified by anion exchange chromatography on an NGC Chromatography system (Bio-Rad Laboratories) using a MonoQ 5/50 GL column (GE Healthcare) and eluting with a 20-600 mM NaCl in 20 mM TRIS (pH 8) gradient. Purified protein was washed and prepared for storage as described above. Protein concentrations were determined from the absorbance at 280 nm measured in Take3 plates on an Epoch2 microplate reader (Biotek) using calculated extinction coefficients (www.benchling.com).

4.2 SDS-PAGE of overexpressed proteins

Purity of proteins was monitored by SDS-PAGE (SI Fig. 6) using Bolt 4-12% Bis-Tris Plus Gels (ThermoFisher Scientific) with MES-SDS running buffer (Novex). Sample load was 0.3-0.6 µg of protein per lane in Bolt LDS sample buffer and Bolt reducing agent. Triple Color Protein Standard III (Serva) was run alongside the protein samples as a size standard. The gels were run at 200 V for 22 minutes and stained with Quick Coomassie stain (Serva).

5 MesG/hydroxylamine spectrophotometric assay

5.1 Michaelis-Menten kinetics of TycA

Michaelis-Menten parameters of the adenylation reaction catalyzed by TycA were determined from kinetic data recorded with the MesG/hydroxylamine assay which was performed as described previously with minor modifications.[16] Reactions contained 50 mM TRIS (pH 7.6), 5 mM $MgCl_2$, 100 µM 7-methylthioguanosine (MesG), 150 mM hydroxylamine (adjusted to pH 7.5-8 with NaOH), 5 mM ATP (A2383, Sigma), 1 mM TCEP, 0.4 U/mL inorganic pyrophosphatase (I1643, Sigma), 1 U/mL of purine nucleoside phosphorylase from microorganisms (N8264, Sigma) and varying amounts of TycA (0.025 – 1 mM) and substrates. In flat-bottom 384-well plates (781620, Brand) 100 µL reactions were started by addition of substrate and the absorbance was followed at 355 nm on a Synergy H1 (BioTek) microplate reader at 30°C. Background activity was recorded in wells containing buffer without substrate and the obtained slopes were subsequently subtracted. Each substrate concentration was measured in duplicate. Initial velocities were divided by the slope of a pyrophosphate calibration curve to obtain the pyrophosphate release rate. Initial velocities $v_0/[E_0]$ were fit to the Michaelis-Menten equation by nonlinear regression using R version 3.4.2 (SI Fig. 2).[17]

5.2 Competitive inhibition of TycA with PheHA

For characterizing competitive inhibition of TycA by PheHA, complete L-Phe kinetic profiles were measured at varying PheHA concentrations (0.74 to 540 µM; Fig. 2B) as described for simple Michaelis-Menten kinetics (Section 5.1). Initial velocities *v* obtained for all combinations of substrate and inhibitor concentrations ([S] and [I]) were fit globally to a competitive inhibition model in R using nonlinear regression and plotted using ggplot2:[17]

```
dat <- read.table("data.csv", sep=';', header=T)      #data input with headers S, I, v
start <- list (kcat=30, Ki=20, Km=0.02)               #starting values (Ki/µM; Km/mM)
f <- v ~ kcat * S / ((1 + I/Ki)*Km + S)               #kinetic model
m <- nls(f,dat,start=start)                           #nonlinear regression
summary(m)                                            #output of fit parameters
```

5.3 TycA stability in 150 mM hydroxylamine

To test the stability of TycA in the presence of 150 mM hydroxylamine, a 10 µM enzyme solution containing 50 mM TRIS (pH 7.6), 5 mM $MgCl_2$ and 150 mM hydroxylamine was incubated at room temperature for up to one hour. After the indicated time, initial adenylation velocities were measured with the preincubated enzyme and three different L-Phe concentrations using the MESG/hydroxylamine assay (SI Fig. 1).

6 Multiplexed hydroxamate assay (HAMA)

6.1 Reaction conditions

The hydroxamate formation assay was conducted at room temperature in 100 µL volume containing 50 mM TRIS (pH 7.6), 5 mM $MgCl_2$, 150 mM hydroxylamine (pH 7.5-8, adjusted with NaOH), 5 mM ATP (A2383, Sigma), 1 mM TCEP and varying concentrations of enzyme. Reactions were started by adding a mix of 5 mM proteinogenic amino acids in 100 mM TRIS (pH 8) to a final concentration of 1 mM or only buffer as a control. For TycA and sdVGrsA assays, L-Phe, L-Val and L-Leu were distinguished from D-Phe, D-Val and L-Ile, respectively by using enantiopure, deuterium labelled standards. Reaction times and temperatures were optimized for each protein. Reactions were quenched at different time points by diluting them 10-fold with 95% acetonitrile in water containing 0.1 % formic acid and submitted to UPLC-MS analysis. Time point t_0 was obtained by quenching the enzyme containing master mix before adding amino acid substrates. To guarantee initial velocity conditions, reactions were quenched before 10% (100 µM) of the most preferred substrate was consumed. We observed a strong impact of sample composition on HILIC separation of hydroxamates. Therefore, care had to be taken that all samples were processed in exactly the same manner without further dilutions, for instance. TycA assays were done in a biological (different enzyme batches) and technical (separate assay reactions) triplicates. Other proteins were assayed from a single protein batch in technical triplicates.

6.2 UPLC-MS/MS conditions

Chromatography was performed on a Waters ACQUITY H-class UPLC system (Waters) with an injection volume of 3 µL. Water with 0.1 % formic acid (A) and acetonitrile with 0.1 % formic acid (B) were used as strong and weak eluent, respectively. Amino acid hydroxamates were separated on the ACQUITY UPLC BEH Amide column (1.7 µm, 2.1 x 50 mm) with a linear gradient of 10-50% A over 5 min (flow rate 0.4 mL/min) followed by 4 min reequilibration. Water containing 0.1% formic acid was used as a needle wash between the samples. Data acquisition and quantitation were done using the MassLynx and TargetLynx software (version 4.1).

MS/MS analyses were performed on Xevo TQ-S micro (Waters) tandem quadrupole instrument with ESI ionisation source in positive ion mode. Nitrogen was used as a desolvation gas and argon as collision gas. The following source parameters were used: capillary voltage 1.5 kV, cone voltage 65 V, desolvation temperature 500°C, desolvation gas flow 1000 L/h. Compounds were detected via specific mass transitions recorded in multiple reaction monitoring (MRM) mode (SI Table 2).

Standard calibration solutions of hydroxamates were prepared ranging from 0.0032 to 10 µM. In general, detection is very sensitive, limits being in the low nanomolar range. However, at such low concentrations, large loss of the linearity of the response was observed. Therefore, here we are defining limits of quantitation (LOQ) as the lowest concentrations of hydroxamate standards at which the signal response was still linear ($R^2 > 0.95$, deviation < 20%). The upper limit of quantification (10 µM) is given by the requirement not to exceed 10% substrate conversion at 1 mM substrate concentration and 10-fold dilution before injection.

6.3 Assay validation with TycA

In order to extend the dynamic range of the assay such that the best six substrates of TycA could be measured across ca. five orders of magnitude in activity (Fig. 2A, SI Table 1), reactions were performed

with and without L-Phe. The PheHA and TrpHA concentrations were determined first by incubation of 1 µM enzyme with complete 1 mM substrate mix (L-Phe-d5, D-Phe, L-Ile, L-Leu-d7, L-Val-d8, D-Val, L-Met, L-Tyr, L-Trp) for 3 min. In the second reaction, 1 µM enzyme was incubated with the same substrate mix lacking L-Phe-d5 and D-Phe for 30 min to allow the accumulation of corresponding hydroxamates up to measurable levels. log([XaaHA]/[TrpHA]) ratios were calculated to allow comparison between both reactions.

6.4 Progress curve of PheHA formation with TycA

A hydroxamate assay reaction with 200 nM TycA in the presence of 1 mM proteinogenic amino acid mix was allowed to run for up to 20 minutes. Reactions were quenched at seven time points and the concentration of PheHA measured (SI Fig. 5).

6.5 Time course of hydroxamate ratios

After a prolonged reaction time, competitive product inhibition will decrease the rate of hydroxamate accumulation,[9] but should not change the ratio of products. Therefore, specificity profiles should remain unaffected. We tested this hypothesis by monitoring hydroxamate ratios over time in the reaction of 1 µM TycA with 1 mM substrates (SI Fig. 4).

7 DKP formation assay

7.1 Reaction conditions

The diketopiperazine (DKP) formation assay was performed in 150 µL volume with 5 mM ATP, 1 mM TCEP, 5 µM GrsB1 and either 5 µM sdVGrsA or a mutant thereof in peptide formation assay buffer (40 mM HEPES, 10 mM $MgCl_2$, 75 mM NaCl, pH 8.0). The reaction was started by addition of L-Val and L-Pro (1 mM each). The resulting solution was incubated at 37 °C and quenched after 3 h by heat denaturation at 95 °C for 3 min. Denatured proteins were precipitated by centrifugation and the supernatant analysed by UPLC-MS/MS.

7.2 UPLC-MS/MS conditions

Chromatography was performed on a Waters ACQUITY H-class UPLC system (Waters) with an injection volume of 2 µL. Methanol (A) and water with 0.1 % formic acid (B) were used as strong and weak eluent, respectively. Diketopiperazines were separated on the ACQUITY UPLC BEH C18 column (1.7 µm, 2.1 x 50 mm) with a linear gradient of 20-60% A over 1.5 min (flow rate 0.5 mL/min) followed by 1 min reequilibration. Acetonitrile was used as a needle wash between the samples. Data acquisition and quantitation were done using the MassLynx and TargetLynx software (version 4.1).

MS/MS analyses were performed on a Xevo TQ-S micro (Waters) tandem quadrupole instrument with ESI ionisation source in positive ion mode. Nitrogen was used as desolvation gas and argon as collision gas. The following source parameters were used: capillary voltage 0.5 kV, cone voltage 4 V, desolvation temperature 600°C, desolvation gas flow 1000 L/h. Val-Pro-DKP and was detected via the 197.09>69.95 transition, recorded in multiple reaction monitoring (MRM) mode. Standard calibration solutions of Val-Pro-DKP were prepared ranging from 0.0006 to 10 µM.

8 Supplementary Tables

SI Table 1. Comparison of kinetic data.*

	PP_i exchange	MesG	HAMA (μM)	
Substrate	k_{cat}/K_M (mM^{-1} min^{-1})[19]	k_{cat}/K_M (mM^{-1} min^{-1})	**3 min**	**30 min**
L-Phe	9900 ± 300	1600 ± 85	96 ± 12	NA
D-Phe	4700 ± 400	2400 ± 120	116 ± 15	NA
L-Tyr	12.2 ± 1.4	1.7 ± 0.3	0.029 ± 0.002	12.7 ± 1.3
L-Trp	5.4 ± 0.5	3.5 ± 0.3	0.13 ± 0.01	43.0 ± 3.0
L-Met	2.1 ± 0.2	3.6 ± 0.8	0.13 ± 0.009	49.0 ± 4.3
L-Leu	1.26 ± 0.1	1.6 ± 0.1	ND	10.9 ± 0.8
L-Val	0.13 ± 0.008	0.12 ± 0.01	ND	0.045 ± 0.008

*NA: Not applicable; ND: Not detectable

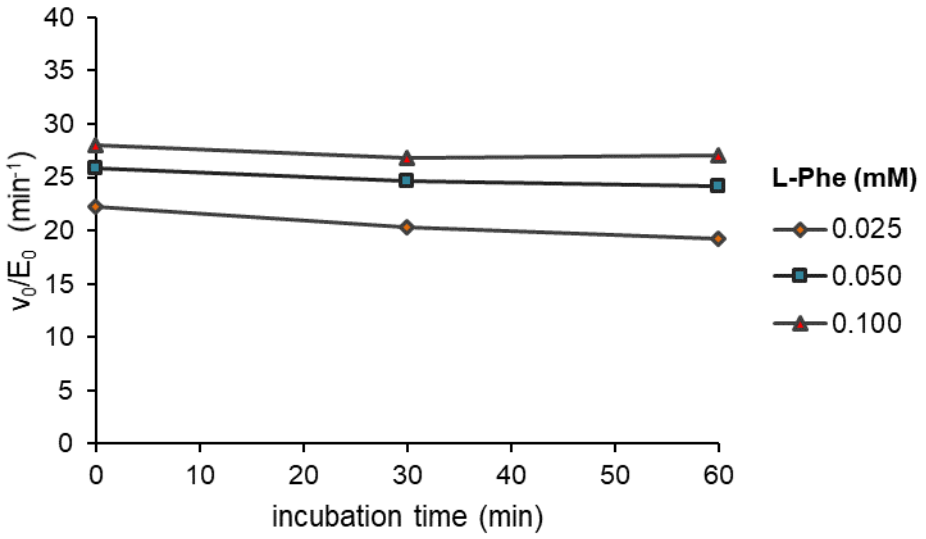
SI Table 2. Acquisition parameters for hydroxamate quantification and limits of quantification (LOQs).

Compound	Parent (m/z)	Cone Voltage (V)	Daughter (m/z)	Collision Energy (V)	LOQ (µM)
AlaHA	104.90	18	43.90	8	0.08
ArgHA	190.02	14	69.94	16	0.016
AspHA	148.95	32	87.92	10	0.08
CysHA	136.87	28	75.87	12	0.0032
GluHA	163.03	24	83.95	18	0.016
GlyHA	90.82	34	29.94	8	0.4
HisHA	171.05	22	109.92	10	0.016
IleHA	147.01	28	85.97	8	0.0032
LysHA	162.02	20	83.94	18	0.08
MetHA	165.03	26	103.88	8	0.0032
D-PheHA	180.99	30	119.94	10	0.0032
ProHA	130.97	24	69.96	12	0.4
ThrHA	134.91	26	73.97	8	0.0032
TrpHA	219.94	30	167.00	16	0.0032
TyrHA	196.98	30	135.95	12	0.0032
D-ValHA	132.87	22	71.91	10	0.016
L-Val-d8-HA	140.92	22	79.96	10	0.016
L-Phe-d5-HA	186.03	30	124.97	10	0.0032
L-Leu-d7-HA	154.05	30	93.01	10	0.0032

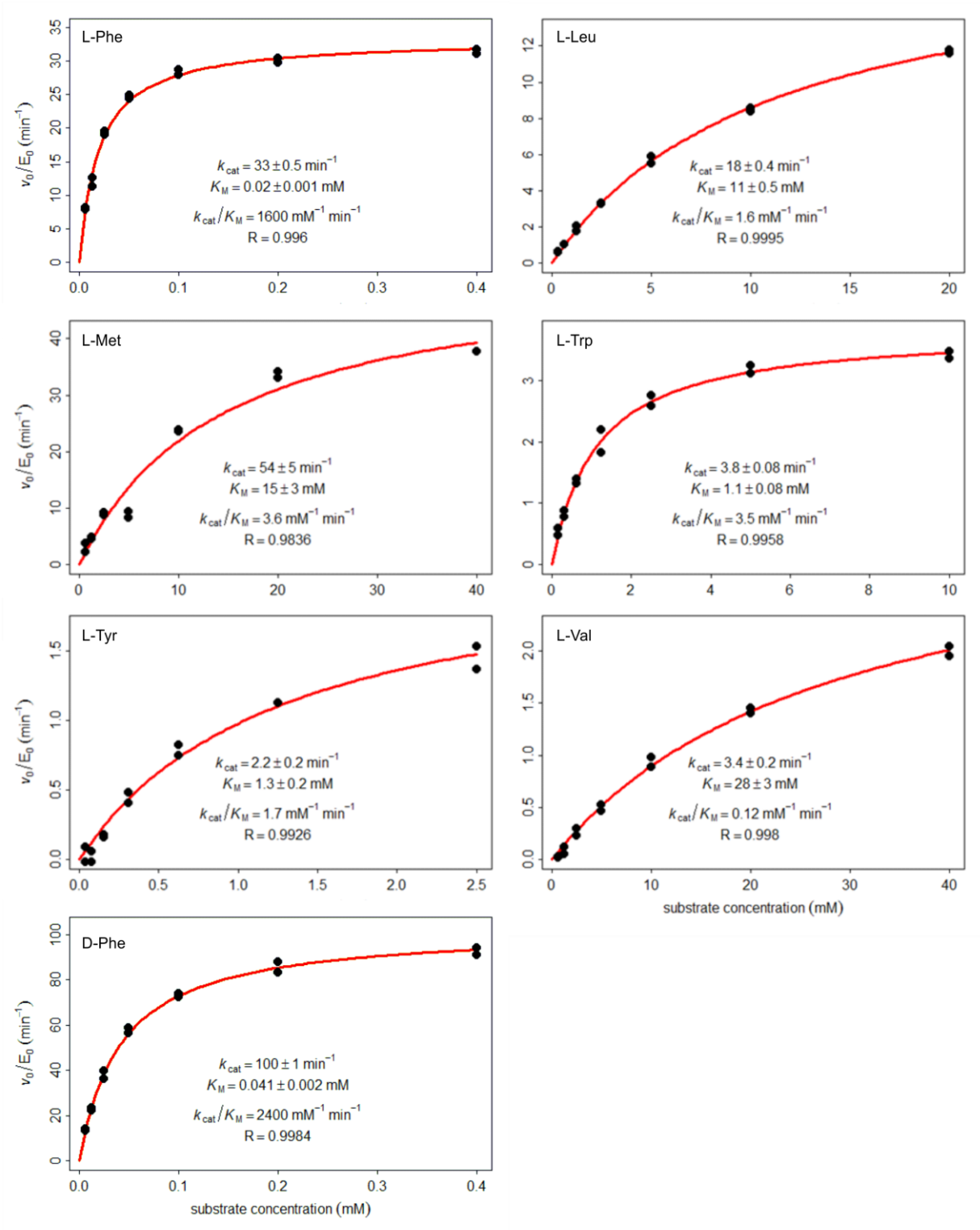
SI Table 3. HRMS of amino acid hydroxamates.

Name	Molecular formula	Expected (m/z)	Found (m/z)	Delta (ppm)
AlaHA	C3H9N2O2+	105.0659	105.0660	1.0
ArgHA	C6H16N5O2+	190.1299	190.1296	1.6
AspHA	C4H9N2O4+	149.0557	149.0555	1.3
CysHA	C3H9N2O2S+	137.0379	137.0378	0.7
GluHA	C5H11N2O4+	163.0713	163.0711	1.2
GlyHA	C2H7N2O2+	91.0502	91.0505	3.3
HisHA	C6H11N4O2+	171.0877	171.0874	1.8
IleHA	C6H15N2O2+	147.1128	147.1126	1.4
LeuHA	C6H15N2O2+	147.1128	147.1126	1.4
LysHA	C6H16N3O2+	162.1237	162.1235	1.2
MetHA	C5H13N2O2S+	165.0692	165.0690	1.2
PheHA	C9H13N2O2+	181.0972	181.0970	1.1
β-PheHA	C9H13N2O2+	181.0972	181.0968	2.2
Phenylglycine HA	C8H11N2O2+	167.0815	167.0813	1.2
Pipecolic acid HA	C6H13N2O2+	145.0972	145.0969	2.1
ProHA	C5H11N2O2+	131.0815	131.0814	0.8
SerHA	C3H9N2O3+	121.0608	121.0608	0.0
ThrHA	C4H11N2O3+	135.0764	135.0763	0.7
TrpHA	C11H14N3O2+	220.1081	220.1079	0.9
TyrHA	C9H13N2O3+	197.0921	197.0919	1.0
ValHA	C5H13N2O2+	133.0972	133.0971	0.8

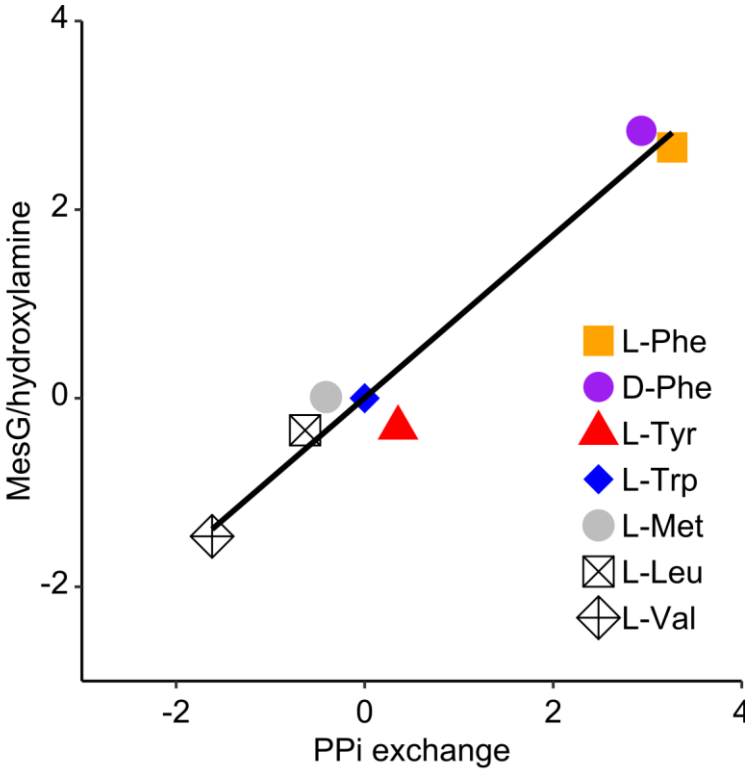
9 Supplementary Figures



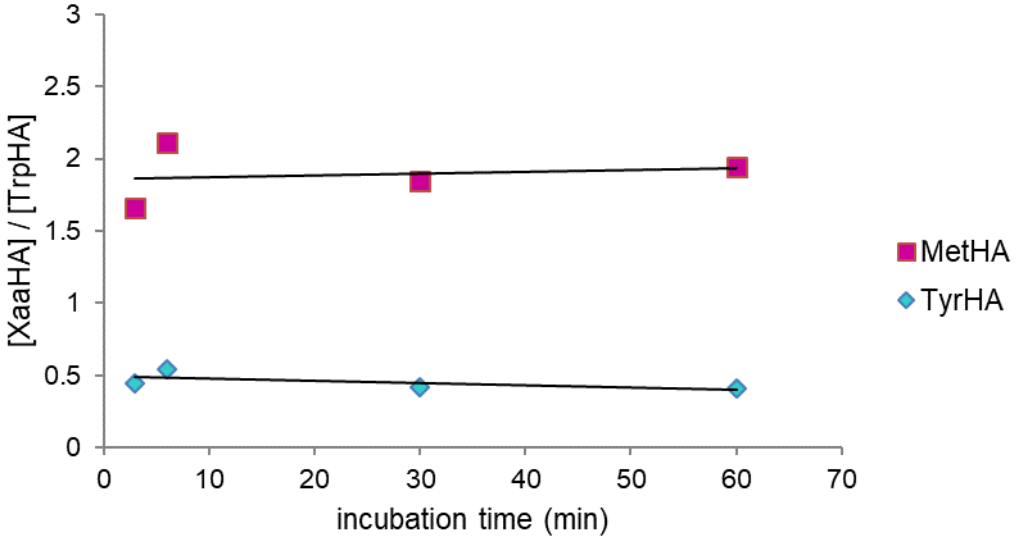
SI Fig. 1. Stability of TycA in hydroxylamine monitored with the MESG/hydroxylamine assay.



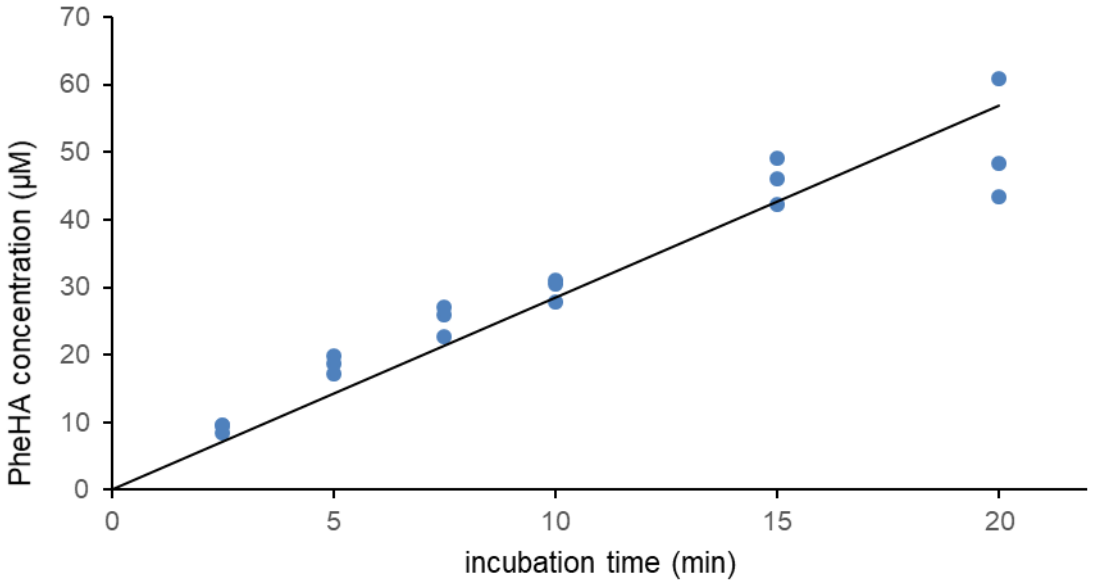
SI Fig. 2. Michaelis-Menten kinetics of TycA.



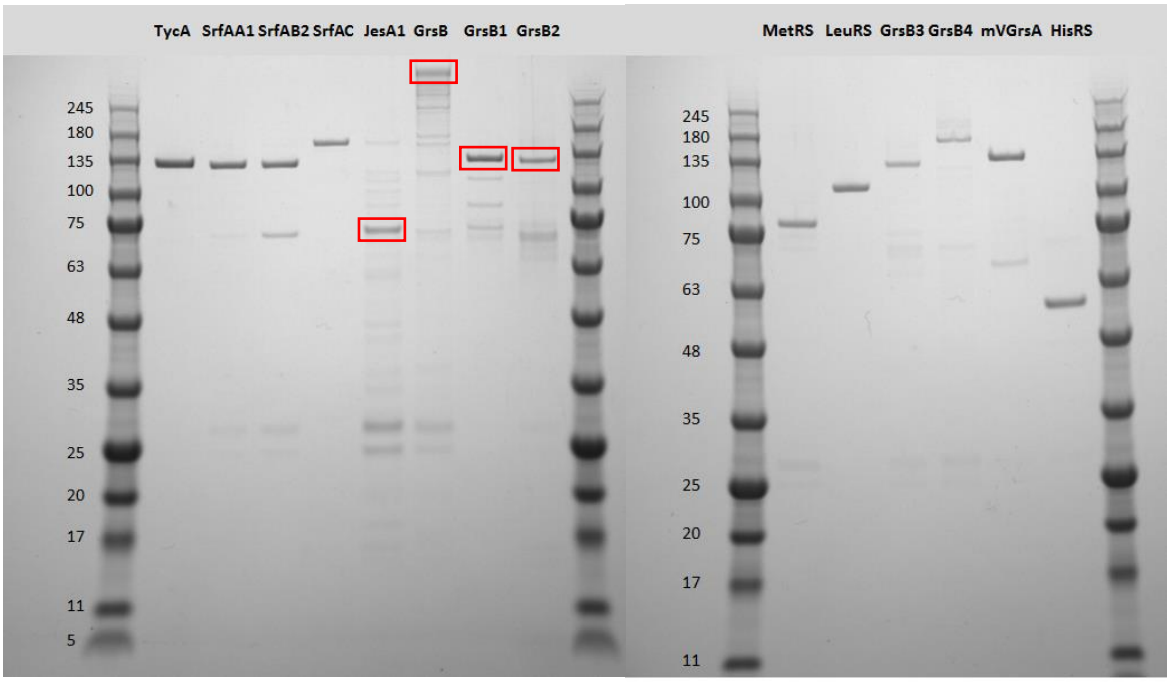
SI Fig. 3. Comparison of TycA parameters measured with MesG/hydroxylamine assay and PPi exchange assay. Data are plotted as log([k_{cat}/K_M]$_{Xaa}$ / [k_{cat}/K_M]$_{Trp}$). Slope: 0.86 ± 0.08; R^2 = 0.957.



SI Fig. 4. Ratios of hydroxamate concentrations during the course of a HAMA assay with TycA. The assay was conducted with the proteinogenic amino acid mix but only MetHA and TyrHA remained in the initial velocity range (<10% conversion) for the entire reaction time.



SI Fig. 5. Progress curve of PheHA formation catalysed by TycA. A linear fit indicates a k_{obs} of 14.2 ± 0.5 min^{-1}. The 3-fold lower turnover rate compared to the k_{cat} determined for pure L-Phe (43 min^{-1}; SI Fig. 2) might be explained by competition with alternative substrates. According to the parameters determined for competitive inhibition (Fig. 2B), the apparent deviation from linearity is not caused by PheHA.



SI Fig. 6. SDS-PAGE of purified proteins. Expected molecular weight of proteins (kDa): TycA (123.6), SrfAA1 (117.1), SrfAB2 (117.0), SrfAC (145.1), JesA1 (67.8), GrsB (510.0), GrsB1 (122.3), GrsB2 (117.9), MetRS (77.2), LeuRS (98.2), GrsB3 (119.3), GrsB4 (152.0), sdVGrsA (128.5), HisRS (48.0). Where ambiguous, the protein of interest is labeled with a red rectangle.

10 Sequences of proteins used in this study

TycA

MVANQANLIDNKRELEQHALVPYAQGKSIHQLFEEQAEAFPDRVAIVFENRRLSYQELNRKANQLARALLEKGVQTDSIVGVMMEKS
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SrfAA1

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H

SrfAB2

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SHHHHHH

SrfAC

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JesA1

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GrsB1

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GrsB2

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GrsB3

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HHHH

GrsB4

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QEDIIVGTPIVGRSHTDLENIVGMFVNTLAMRNKPEGEKTFKAFVSEIKQNALAAFENQDYPFEELIEKLEIQRDLSRNPLFDTLFS
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GrsB

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MetRS

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HKVNPFKALYNRIDMRQVEALVEASKEEVKAAAAPVTGPLADDPIQETITFDDFAKVDLRVALIENAEFVEGSDKLLRLTLDLGGEK
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LeuRS

MQEQYRPEEIESKVQLHWDEKRTFEVTEDESKEKYYCLSMLPYPSGRLHMGHVRNYTIGDVIARYQRMLGKNVLQPIGWDAFGLPAE
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AAGHPLAQKAAENNPELAAFIDECRNTKVAEAEMATMEKKGVDTGFKAVHPLTGEEIPVWAANFVLMEYGTGAVMAVPGHDQRDYEF
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TLEDGTVMPTPDDQLPVILPEDVVMDGITSPIKADPEWAKTTVNGMPALRETDTFDTFMESSWYYARYTCPQYKEGMLDSEAANYWL
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NAPWPVADEKAMVEDSTLVVVQVNGKVRAKITVPVDATEEQVRERAGQEHLVAKYLDGVTVRKVIYVPGKLLNLVVGKHHHHHH

HisRS

MAKNIQAIRGMNDYLPGETAIWQRIEGTLKNVLGSYGYSEIRLPIVEQTPLFKRAIGEVTDVVEKEMYTFEDRNGDSLTLRPEGTAG
CVRAGIEHGLLYNQEQRLWYIGPMFRHERPQKGRYRQFHQLGCEVFGLQGPDIDAELIMLTARWWRALGISEHVTLELNSIGSLEAR
ANYRDALVAFLEQHKEKLDEDCKRRMYTNPLRVLDSKNPEVQALLNDAPALGDYLDEESREHFAGLCKLLESAGIAYTVNQRLVRGL
DYYNRTVFEWVTNSLGSQGTVCAGGRYDGLVEQLGGRATPAVGFAMGLERLVLLVQAVNPEFKADPVVDIYLVASGADTQSAAMALA
ERLRDELPGVKLMTNHGGGNFKKQFARADKWGARVAVVLGESEVANGTAVVKDLRSGEQTAVAQDSVAAHLRTLLGKHHHHHH

sdV-GrsA

MLNSSKSILIHAQNKNGTHEEEQYLFAVNNTKAEYPRDKTIHQLFEEQVSKRPNNVAIVCENEQLTYHELNVKANQLARIFIEKGIG
KDTLVGIMMEKSIDLFIGILAVLKAGGAYVPIDIEYPKERIQYILDDSQARMLLTQKHLVHLIHNIQFNGQVEIFEEDTIKIREGTN
LHVPSKSTDLAYVIYTSGTTGNPKGTMLEHKGISNLKVFFENSLNVREDDRIIQTGAIGFDALTFEVFGSLLHGAELYPVTKDVLLD
AEKLHKFLQANQITIMWLTSPLFNQLSQGTEEMFAGLRSLIVGGDALSPKHINNVKRKCPNLTMWNGYGPTENTTFSTCFLIDKEYD
DNIPIGKAIQNTQIYIVDENLQLKSVGEAGELCIGGEGLARGYWKRPELTSQKFVDNPFVPGEKLYKTGDQARWLSDGNIEYLGRID
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QTIALPEKTDSFKDWSIELEKYANSELFLEEAEYWHHLNYYTENVQIKKDYVTMNNKQKNIRYVGMELTIEETEKLLKNVNKAYRTE
INDILLTALGFALKEWADIDKIVINLEGHGREEILEQMNIARTVGWFTSQYPVVLDMQKSDDLSYQIKLMKENLRRIPNKGIGYEIF
KYLTTEYLRPVLPFTLKPEINFNYLGQFDTDVKTELFTRSPYSMGNSLGPDGKNNLSPEGESYFVLNINGFIEEGKLHITFSYNEQQ
YKEDTIQQLSRSYKQHLLAIIEHCVQKEDTELTPSDFSFKELELEEMDDIFDLLADSLTGSRSHHHHHH

11 NMR analysis and spectra

NMR measurements were performed on a Bruker AVANCE II 300 MHz, Bruker AVANCE II 500 MHz and a Bruker AVANCE II 600 MHz spectrometer, equipped with a Bruker Cryoplatform. The chemical shifts are reported in parts per million (ppm) relative to the solvent residual peak of D_2O (^1H: 4.79 ppm, singlet) for ^1H and trifluoroacetic acid (^{13}C: 164.2 ppm, quartet) for ^{13}C spectra. For NMR analysis, hydroxamates and corresponding amino acids were dissolved in 1.8% trifluoroacetic acid (TFA) in D_2O and recorded NMR spectra were compared. The conversion to hydroxamic acid is determined by ~0.2 ppm shift of Cα ^1H and ~5 ppm shift of $^{13}C\alpha$ with respect to the corresponding proton and carbon shifts of free amino acid. The purity of hydroxamates was determined by comparing integral of Cα ^1H of the hydroxamate to the $^{13}C\alpha$ proton of corresponding free amino acid, which was a major impurity. Atoms are labeled according to the atom names, remoteness codes and order indicators for amino acid residues of Protein Data Bank (PDB) nomenclature.

SI Table 4. NMR data.

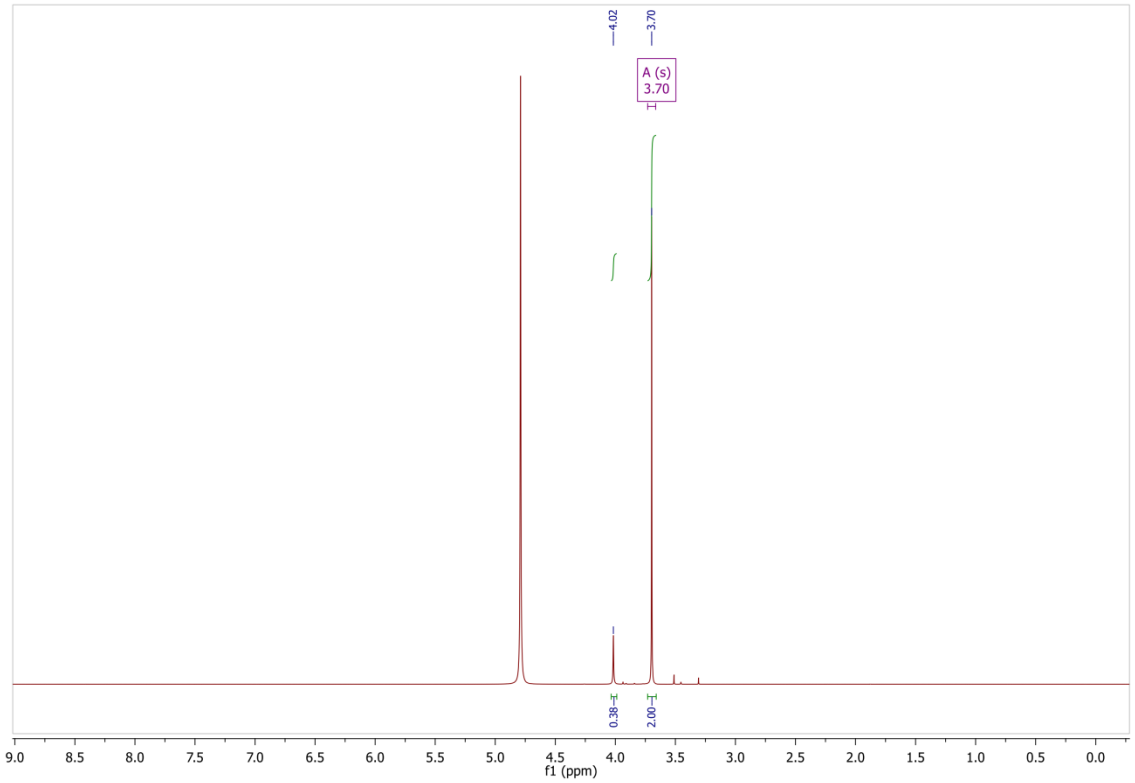
Compound	Position	δ_H, mult. (*J* in Hz)/nH	δ_C
GlyHA	C		165.7
	Cα	3.70, s/1H	39.7
AlaHA	C		169.1
	Cα	3.94, q (7.1)/1H	48.5
	Cβ	1.46, d (7.1)/3H	17.7
SerHA	C		166.3
	Cα	4.05, dd (5.9, 4.7)/1H	54.0
	Cβ	3.96, dd (12.3, 4.7)/1H 3.90, dd (12.3, 5.9)/1H	61.4
ThrHA	C		166.2
	Cα	3.68, d (7.2)/1H	58.5
	Cβ	4.12 – 4.05, m/1H	67.6
	Cγ	1.26, d (6.4)/3H	20.2
CystineHA	C		166.1
	Cα	4.23, dd (6.8)/1H	51.4
	Cβ	3.31, dd (14.8, 6.5)/1H 3.23, dd (14.8, 7.2)/1H	38.5
ValHA	C		167.4
	Cα	3.53, d (7.1)/1H	58.1
	Cβ	2.16 – 2.02, m/1H	31.2
	Cγ1	0.98, d (6.8)/3H	18.8
	Cγ2	0.93, d (6.8)/3H	18.8
LeuHA	C		168.4
	Cα	3.80, dd (7.4)/1H	51.2
	Cβ	1.75 – 1.50, m/3H	40.9
	Cγ		25.3
	Cδ1	0.88, d (2.7)/3H	22.7
	Cδ2	0.86, d (2.6)/3H	22.6
IleHA	C		167.5
	Cα	3.61, d (6.8)/1H	57.0
	Cβ	1.93 – 1.80, m/1H	37.5
	Cγ1	1.52 – 1.39, m/1H 1.26 – 1.09, m/1H	25.9
	Cγ2	0.96 – 0.81, m/6H	15.2
	Cδ		11.6
MetHA	C		167.5
	Cα	3.97, dd (7.1)/1H	51.6
	Cβ	2.63 – 2.50, m/2H	31.0
	Cγ	2.17 – 2.10, m/2H	29.6
	Cε	2.08, s/3H	15.3

SI Table 4. NMR data (continued).

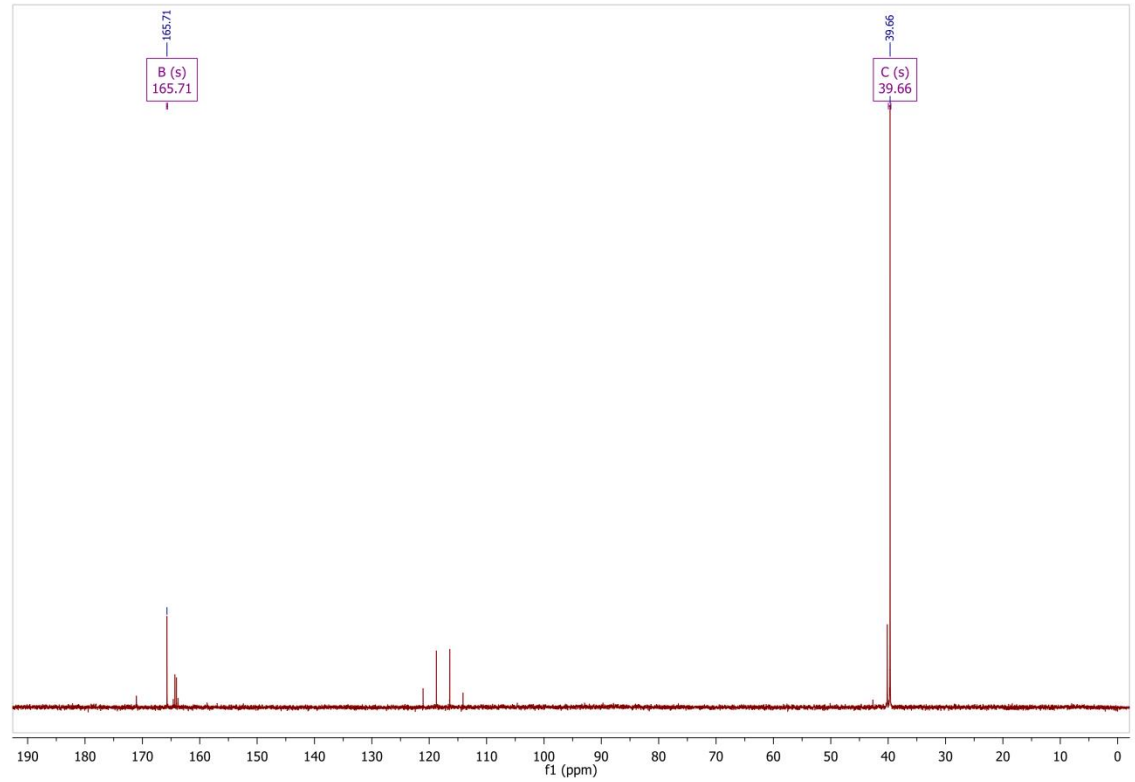
Compound	Position	δ_H, mult. (*J* in Hz)/nH	δ_C
ProHA	C		168.2
	Cα	4.25, dd (7.6)/1H	59.3
	Cβ	2.43 – 2.34, m/1H	31.0
	Cγ	2.10 – 2.01, m/3H	25.3
	Cδ	3.46 – 3.35, m/2H	48.0
PheHA (DMSO-d_6)	C		164.2
	Cα	3.86 – 3.67, m/1H	51.8
	Cβ	3.03 – 2.96, m/2H	37.1
	Cγ		135.0
	Cδ1	7.39- 7.16, m/5H	128.7
	Cδ2		
	Cε1		129.5
	Cε2		
	Cζ		127.3
TyrHA	C		167.2
	Cα	3.95, dd (6.8, 8.3)/1H	54.1
	Cβ	3.11 – 3.00, m/2H	37.3
	Cγ		126.9
	Cδ1	7.13 – 7.08, m/2H	132.2
	Cδ2		
	Cε1	6.87 – 6.82, m/2H	117.2
	Cε2		
	Cζ		156.5
TrpHA	C		167.8
	Cα	4.07, dd (7.4)/1H	53.2
	Cβ	3.33, d (3.0)/1H 3.31, d (2.2)/1H	28.3
	Cγ		107.8
	Cδ1	7.26, s/1H	126.8
	Cδ2		127.9
	Cε2		137.7
	Cε3	7.61 – 7.57, m/1H	119.6
	Cζ2	7.5 – 7.46, m/1H	113.5
	Cζ3	7.24 – 7.21, m/1H	121.0
	Cη2	7.17 – 7.11, m/1H	123.6
AspHA	C		166.9
	Cα	4.26, dd (6.5)/1H	49.0
	Cβ	3.08 – 2.96, m/2H	36.0
	Cγ		173.7
GluHA	C		167.4
	Cα	3.93, dd (7.0)/1H	51.9
	Cβ	2.16, m/2H	27.1
	Cγ	2.53, dd (12.3, 7.2)/2H	30.4
	Cδ		177.3

SI Table 4. NMR data (continued).

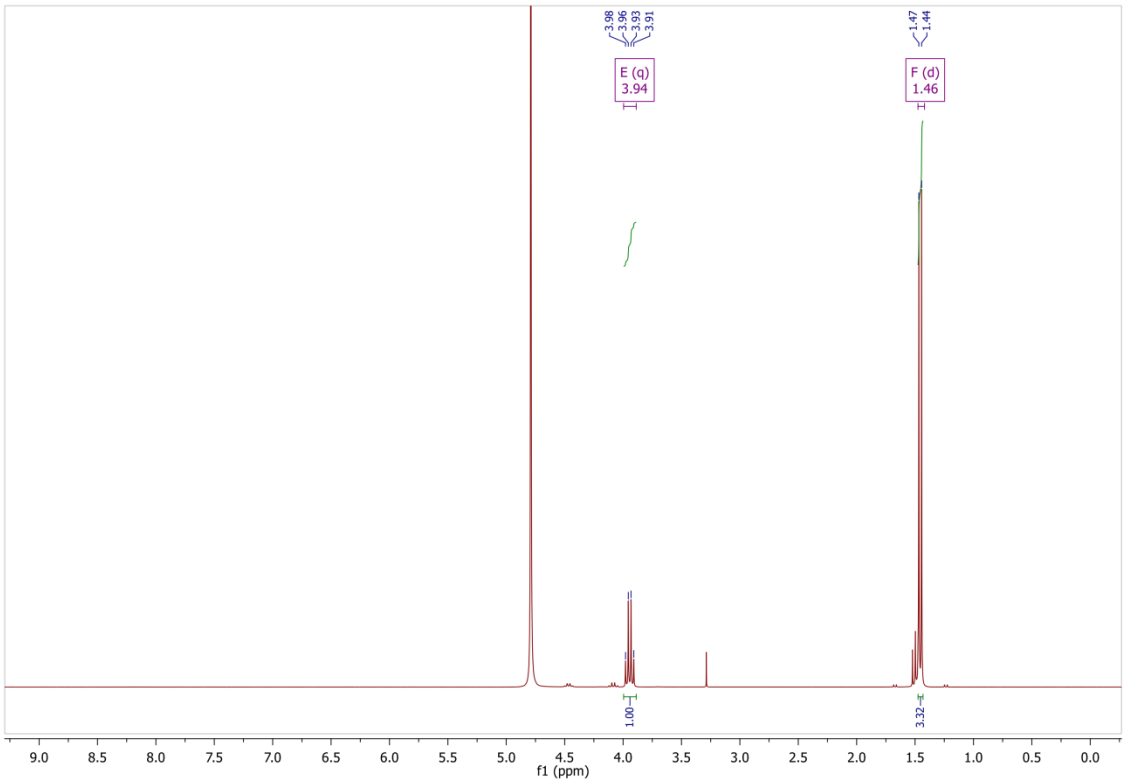
Compound	Position	δ_H, mult. (*J* in Hz)/nH	δ_C
HisHA	C		166.1
	Cα	4.15, dd (7.3)/1H	51.7
	Cβ	3.44 – 3.35, m/2H	27.2
	Cγ		127.1
	Cδ2	7.44, s/1H	119.8
	Cε1	8.72, s/1H	135.8
LysHA	C		167.8
	Cα	3.79, dd (7.1)/2H	52.4
	Cβ	1.89 – 1.79, m/2H	31.5
	Cγ	1.43 – 1.33, m/2H	22.7
	Cδ	1.70 – 1.58, m/2H	27.6
	Cε	2.98 – 2.89, m/2H	40.4
ArgHA	C		167.7
	Cα	3.86, dd (7.0)/1H	52.4
	Cβ	1.93 – 1.86, m/2H	29.3
	Cγ	1.67 – 1.58, m/2H	25.2
	Cδ	3.24 – 3.18, m/2H	41.8
	Cζ		65.8
PipHA	C		168.1
	Cα	3.84, dd (12.0, 3.4)/1H	57.0
	Cβ	2.13 – 2.03, m/1H 1.97 – 1.47, m/5H	28.1
	Cγ		22.3
	Cδ		22.4
	Cε	3.54 – 3.39, m/1H 3.12 – 2.98, m/1H	45.3
Phenylglycine HA	C		167.1
	Cα	4.97, s/1H	55.8
	Cβ	7.44 – 7.34, m/5H	132.8
	Cγ1		131.7
	Cγ2		
	Cδ1		130.9
	Cδ2		
	Cε		129.1
β-PheHA	C		169.0
	Cα	2.82, dd (14.9, 6.7)/1H 2.68, dd (14.9, 8.0)/1H	37.8
	Cβ	4.65 – 4.53, dd (7.3)/1H	53.6
	Cγ	7.38 – 7.24, m/5H	136.0
	Cδ1		130.8
	Cδ2		
	Cε1		131.1
	Cε2		
	Cζ		128.3



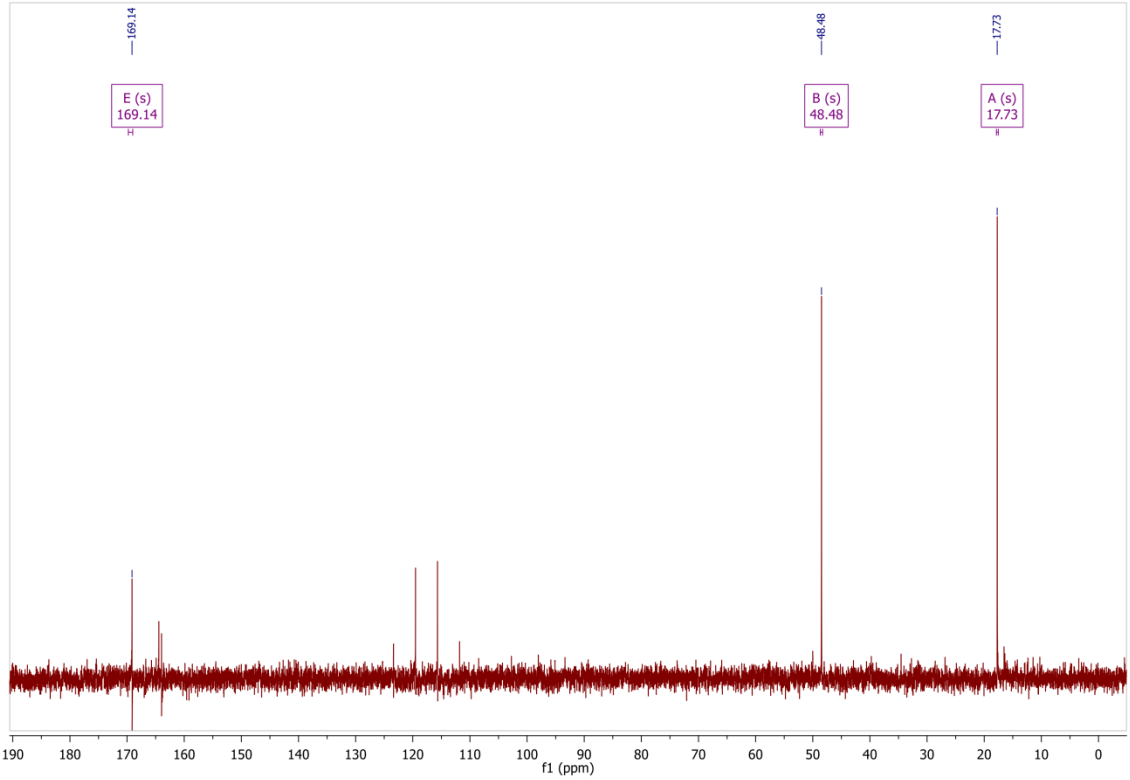
^{1}H NMR spectrum of GlyHA (D_2O + 1.8% TFA, 300 MHz).



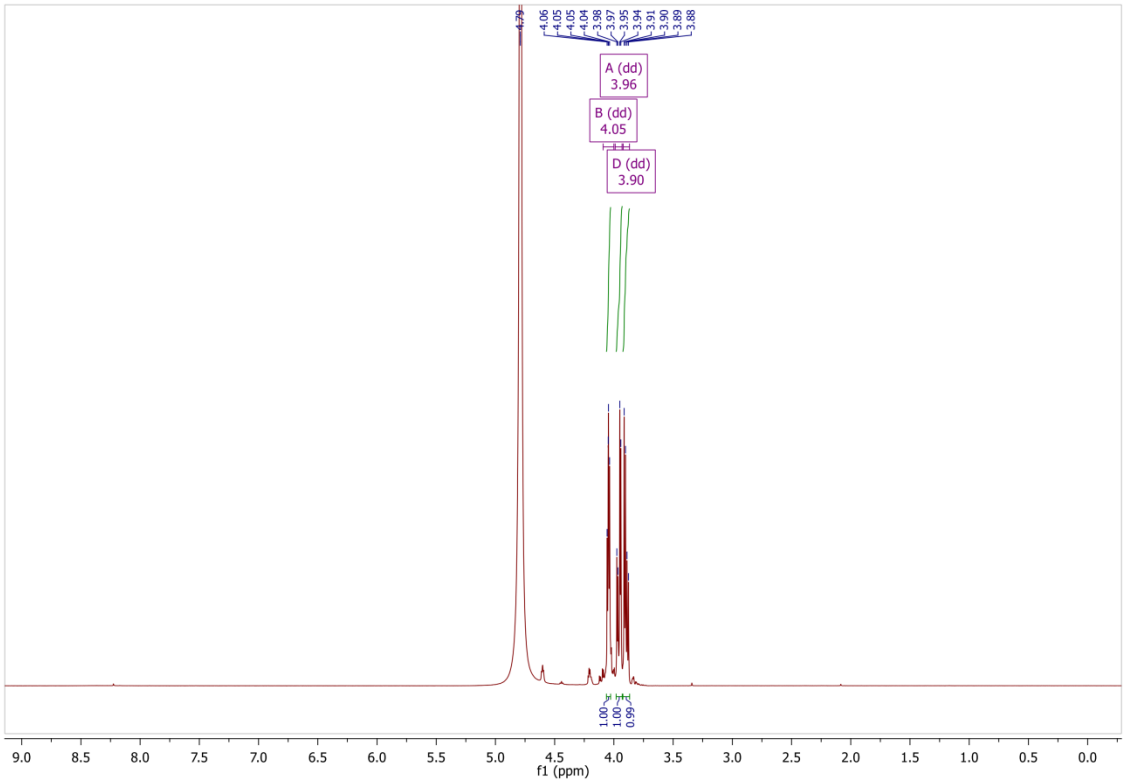
^{13}C NMR spectrum of GlyHA (D_2O + 1.8% TFA, 126 MHz).



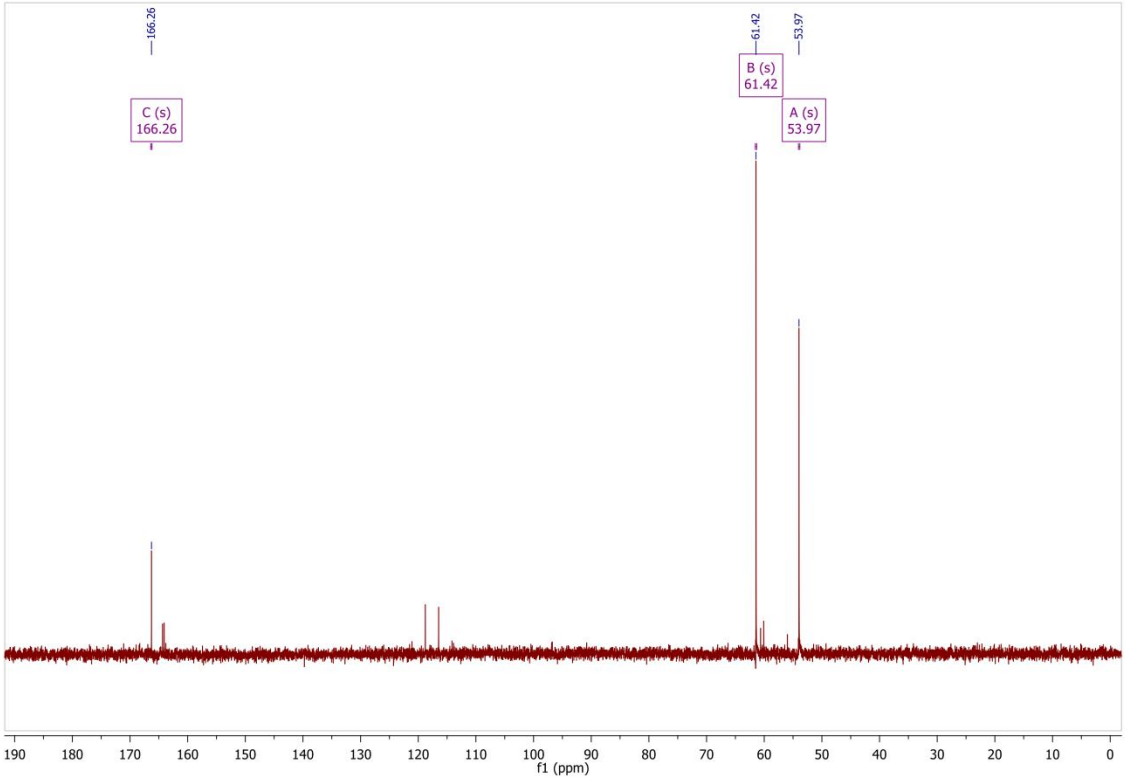
^{1}H NMR spectrum of AlaHA (D_2O + 1.8% TFA, 300 MHz).



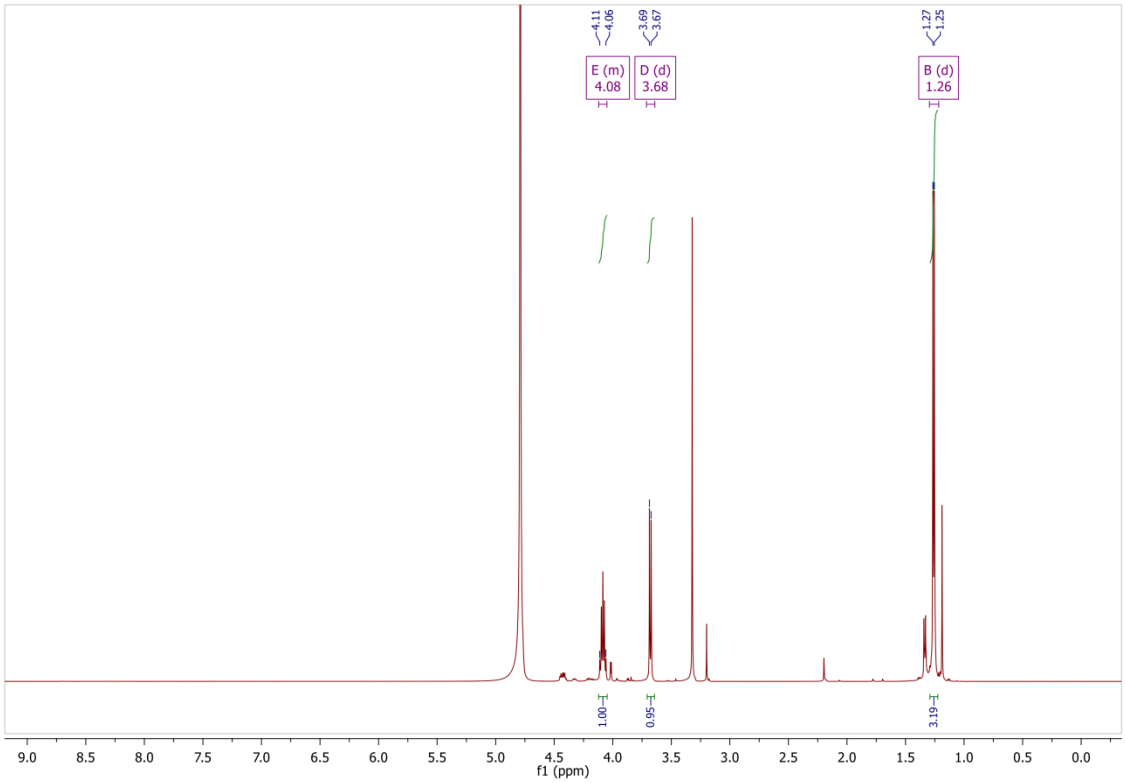
^{13}C NMR spectrum of AlaHA (D_2O + 1.8% TFA, 75 MHz).



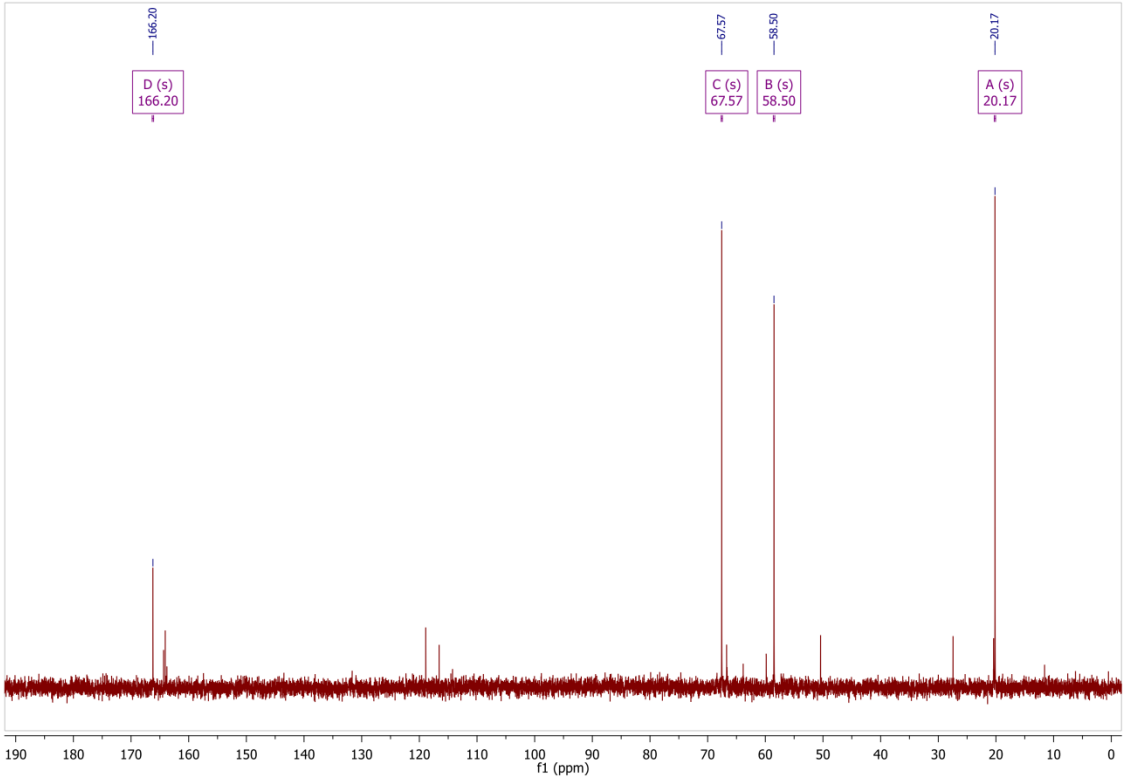
^{1}H NMR spectrum of SerHA (D_2O + 1.8% TFA, 500 MHz).



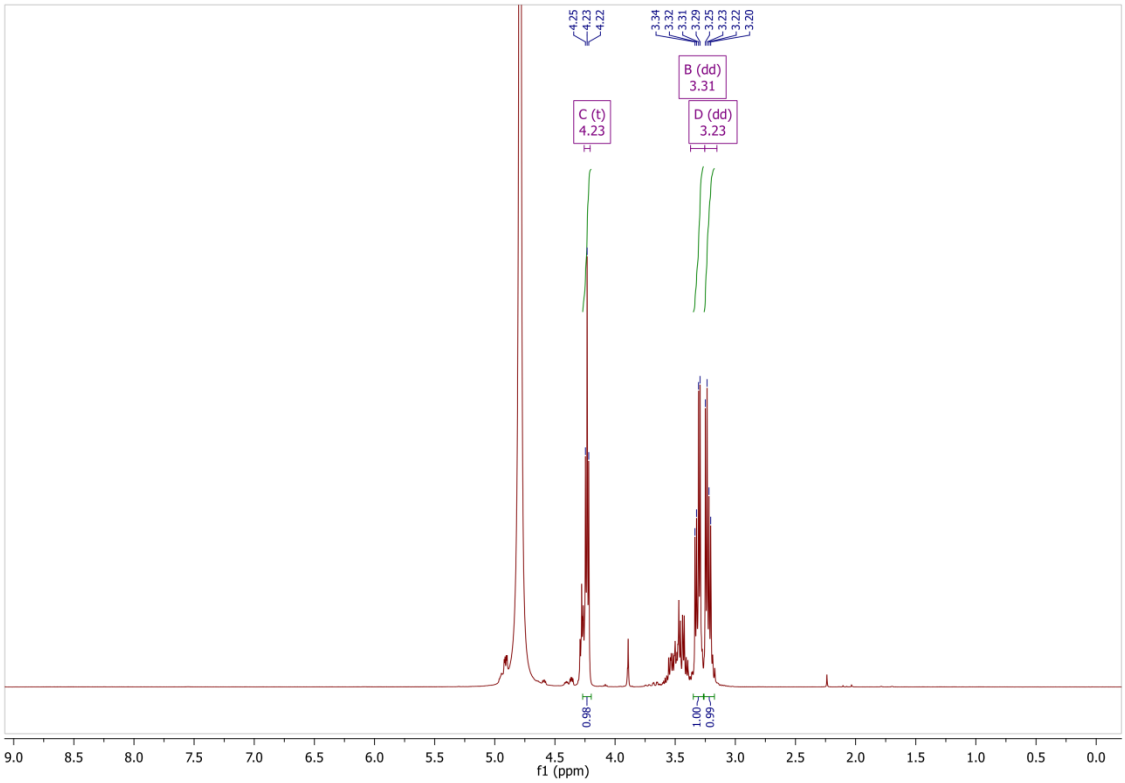
^{13}C NMR spectrum of SerHA (D_2O + 1.8% TFA, 126 MHz).



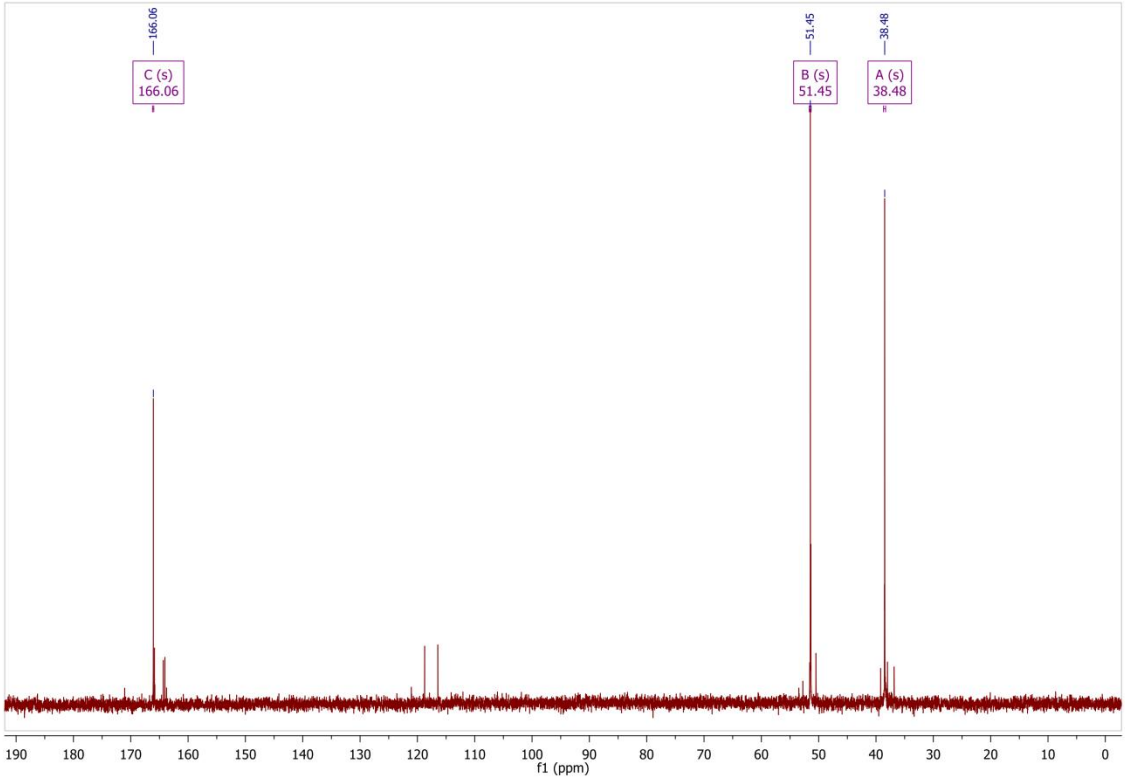
^{1}H NMR spectrum of ThrHA (D_2O + 1.8% TFA, 500 MHz).



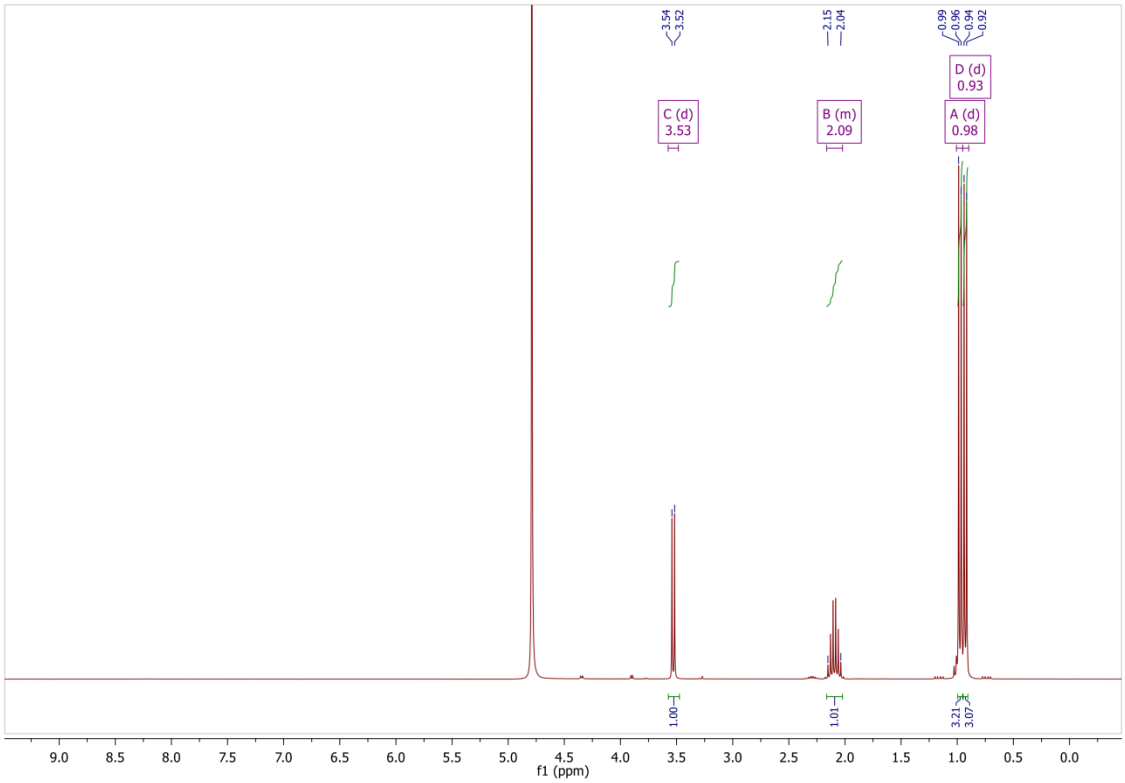
^{13}C NMR spectrum of ThrHA (D_2O + 1.8% TFA, 126 MHz).



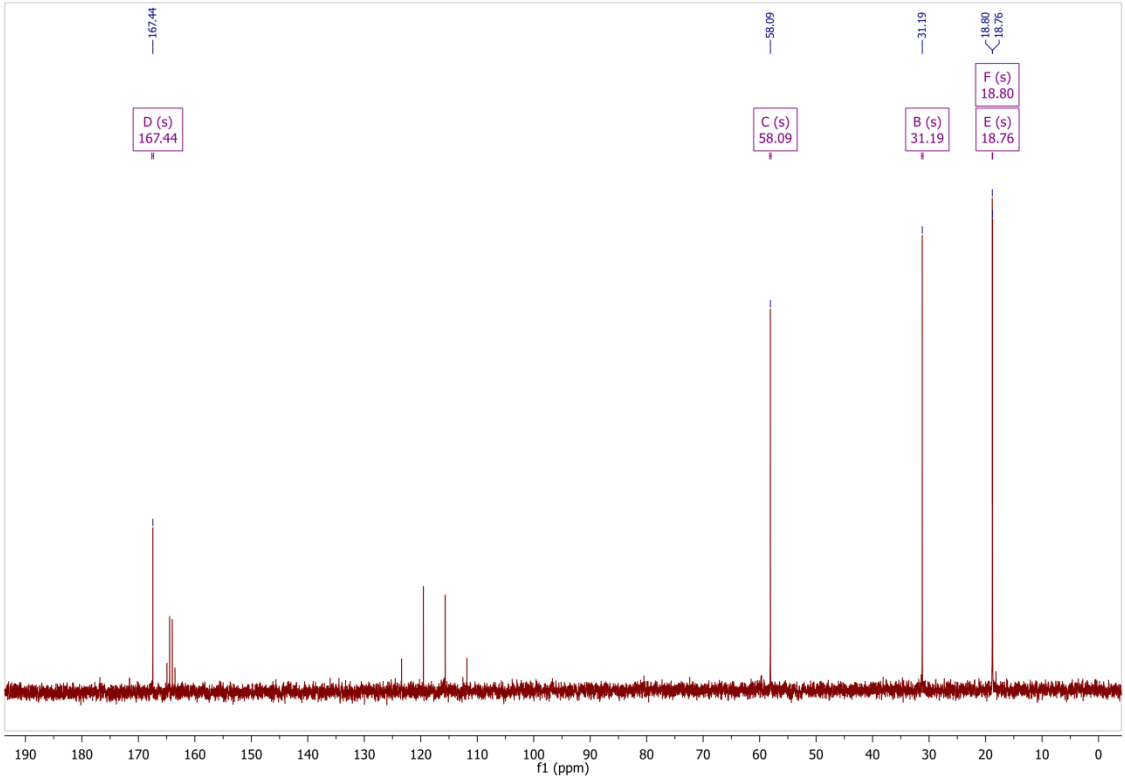
^{1}H NMR spectrum of cystine hydroxamate (D_2O + 1.8% TFA, 500 MHz).



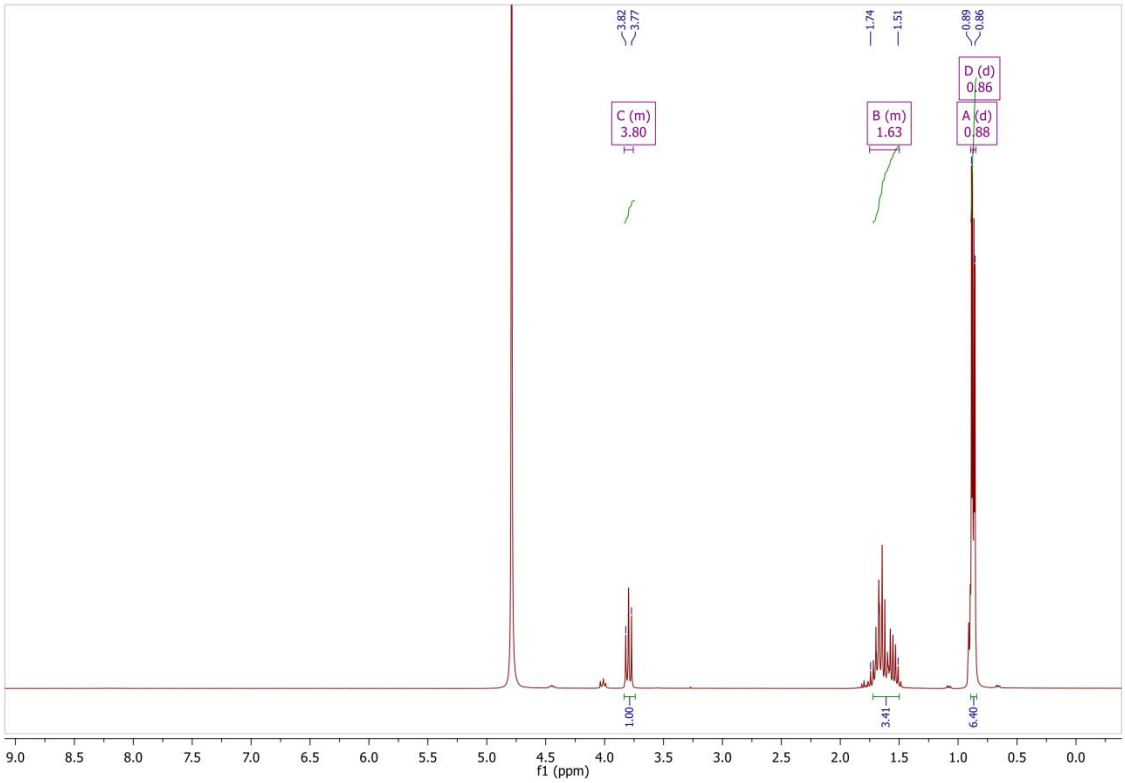
^{13}C NMR spectrum of cystine hydroxamate (D_2O + 1.8% TFA, 126 MHz).



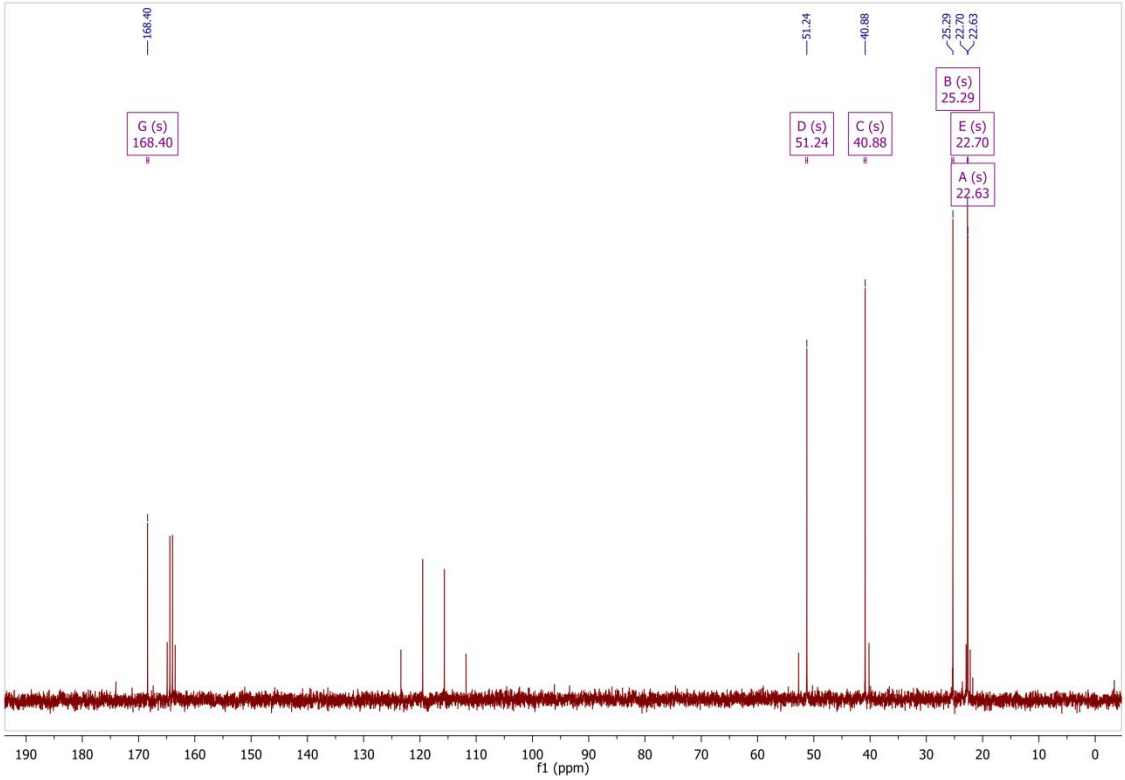
^{1}H NMR spectrum of ValHA (D_2O + 1.8% TFA, 300 MHz).



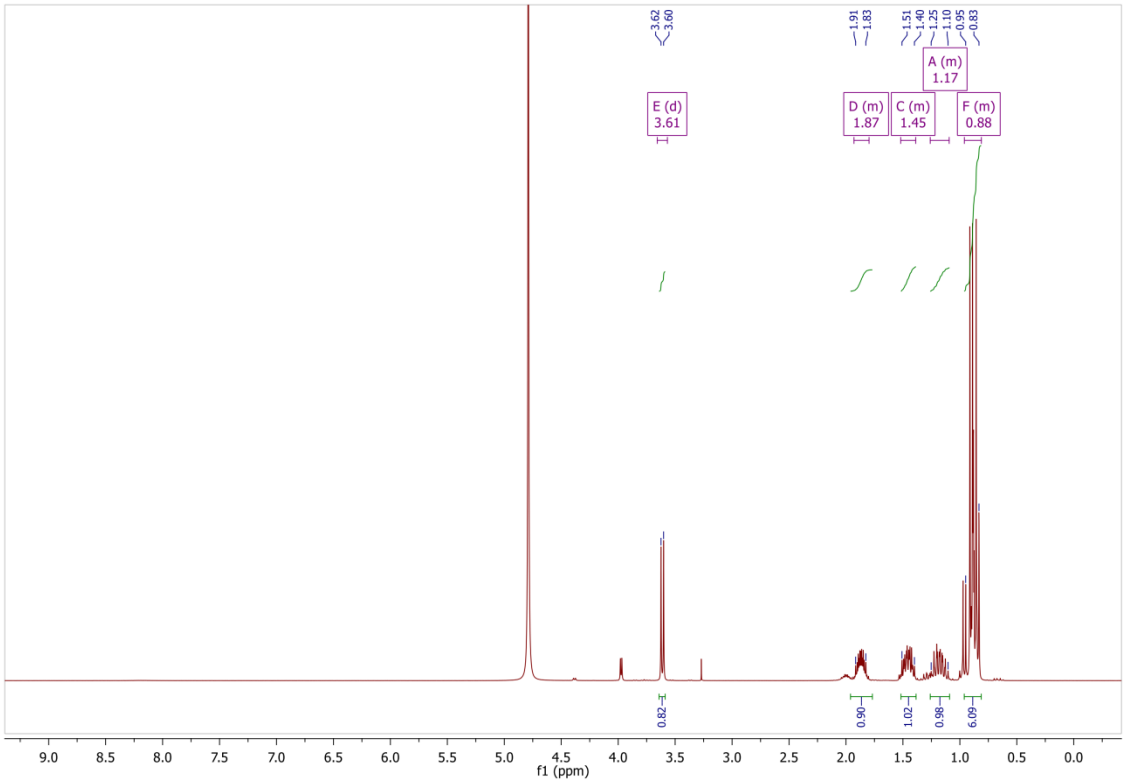
^{13}C NMR spectrum of ValHA (D_2O + 1.8% TFA, 75 MHz).



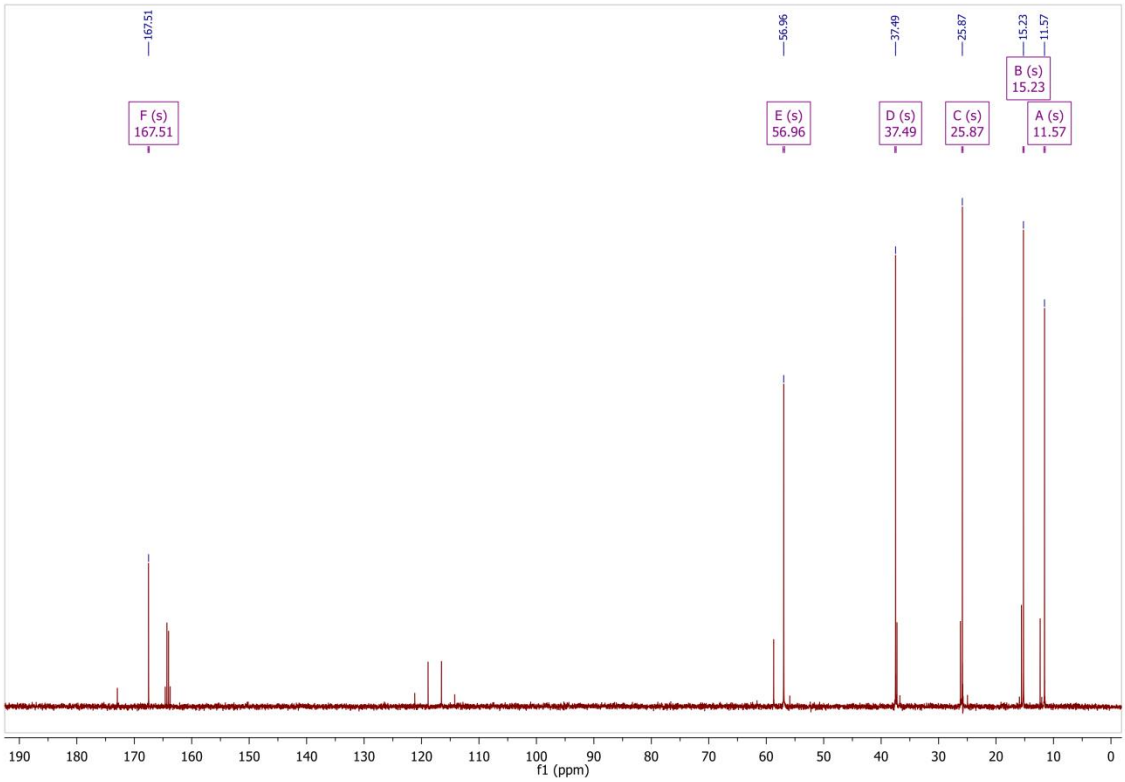
^{1}H NMR spectrum of LeuHA (D_2O + 1.8% TFA, 300 MHz).



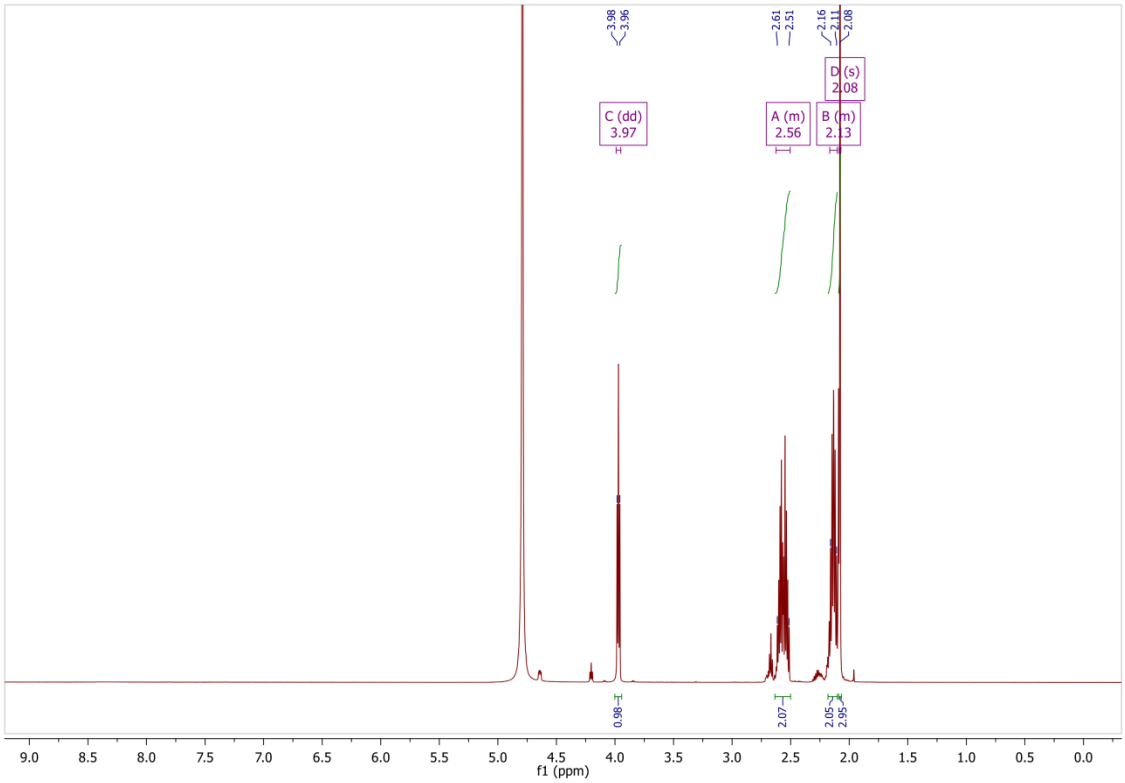
^{13}C NMR spectrum of LeuHA (D_2O + 1.8% TFA, 75 MHz).



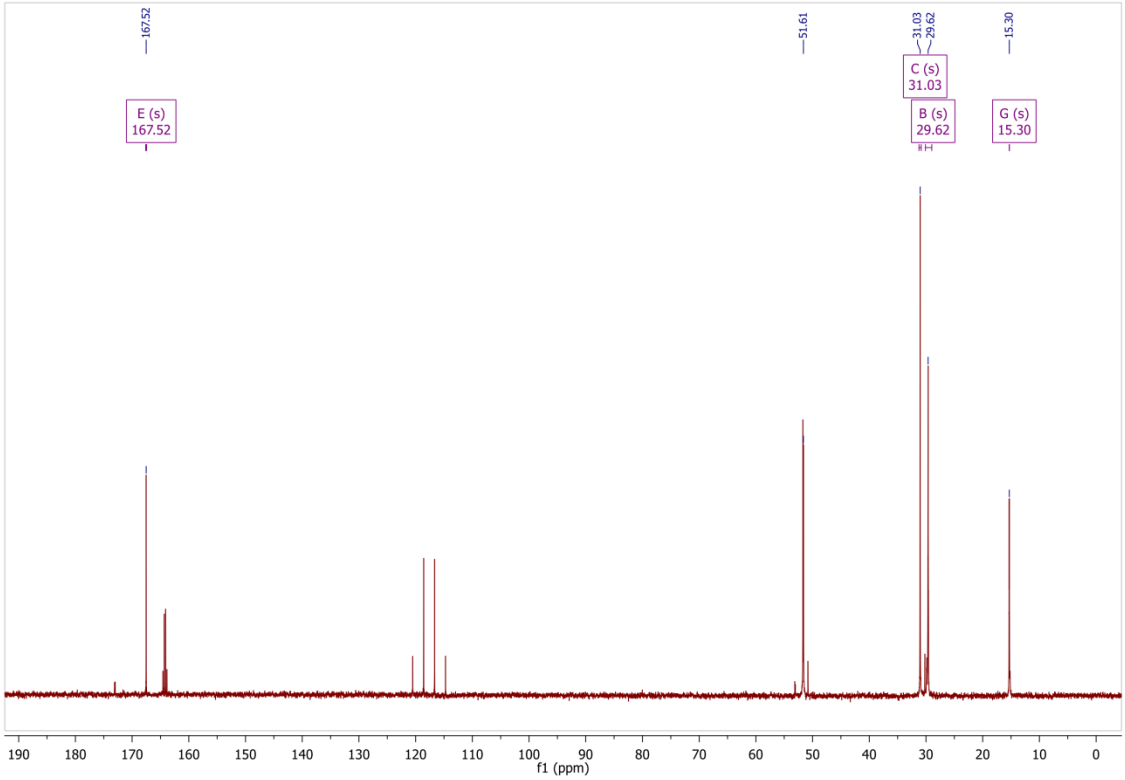
^{1}H NMR spectrum of IleHA (D_2O + 1.8% TFA, 300 MHz).



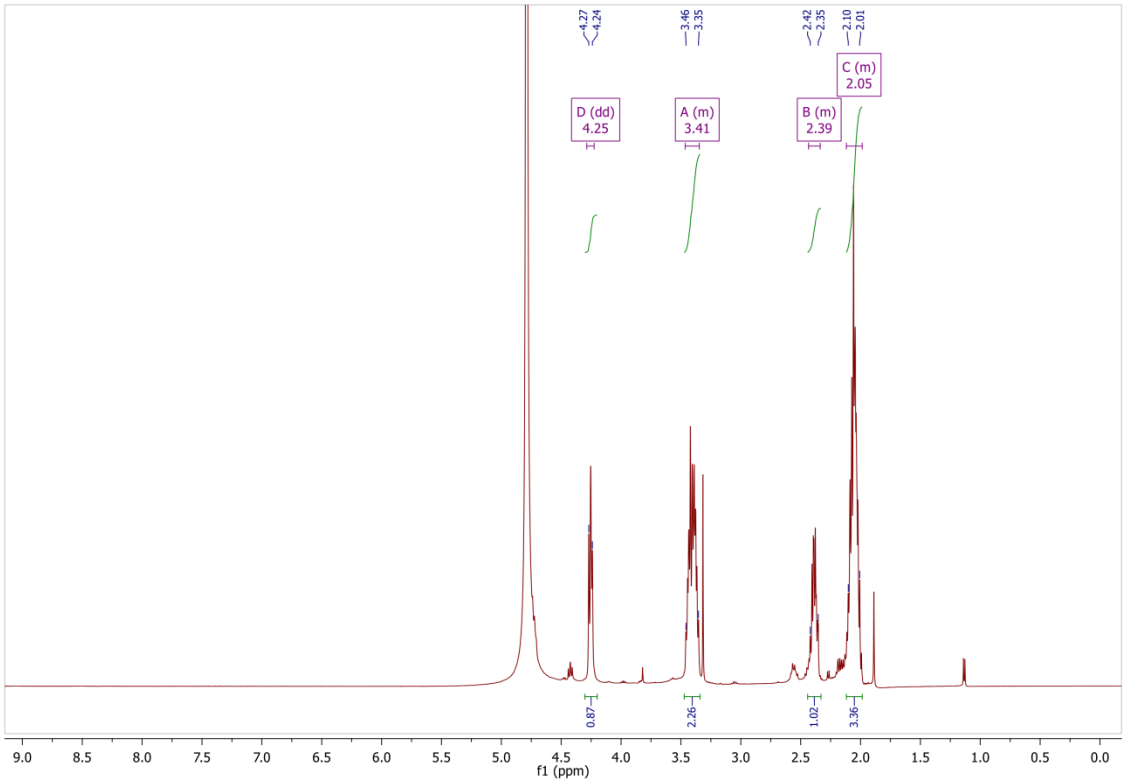
^{13}C NMR spectrum of IleHA (D_2O + 1.8% TFA, 126 MHz).



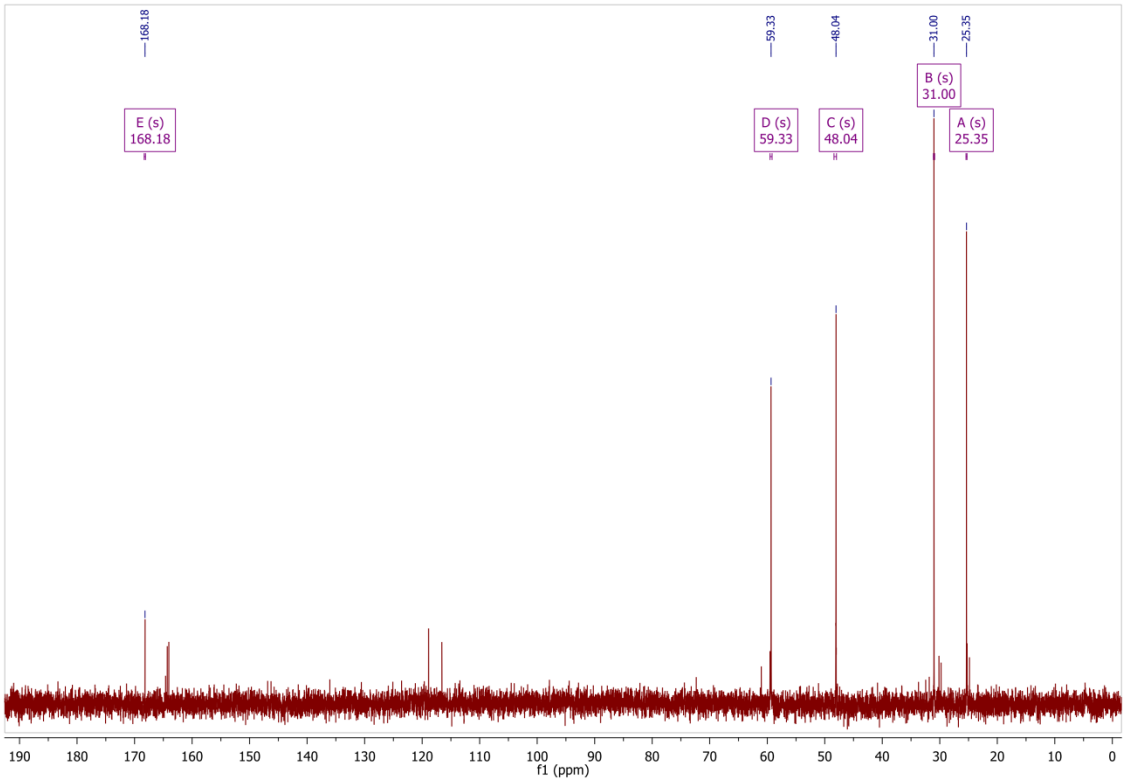
^{1}H NMR spectrum of MetHA (D_2O + 1.8% TFA, 600 MHz).



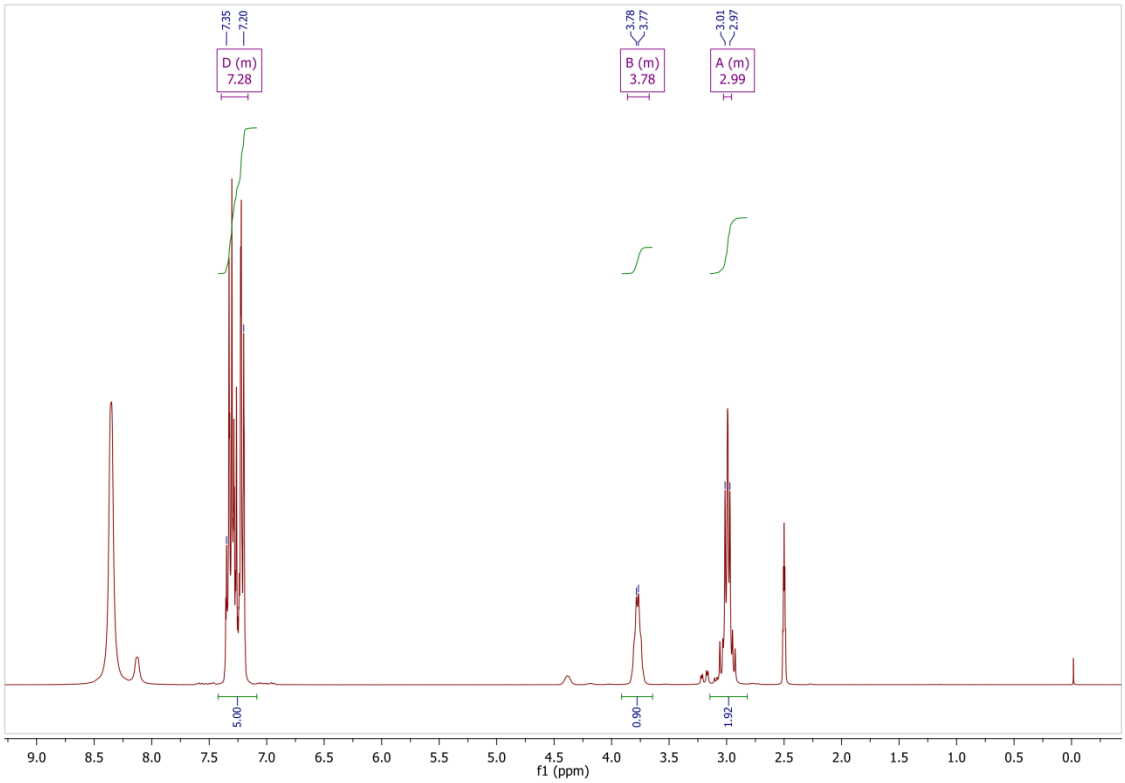
^{13}C NMR spectrum of MetHA (D_2O + 1.8% TFA, 151 MHz).



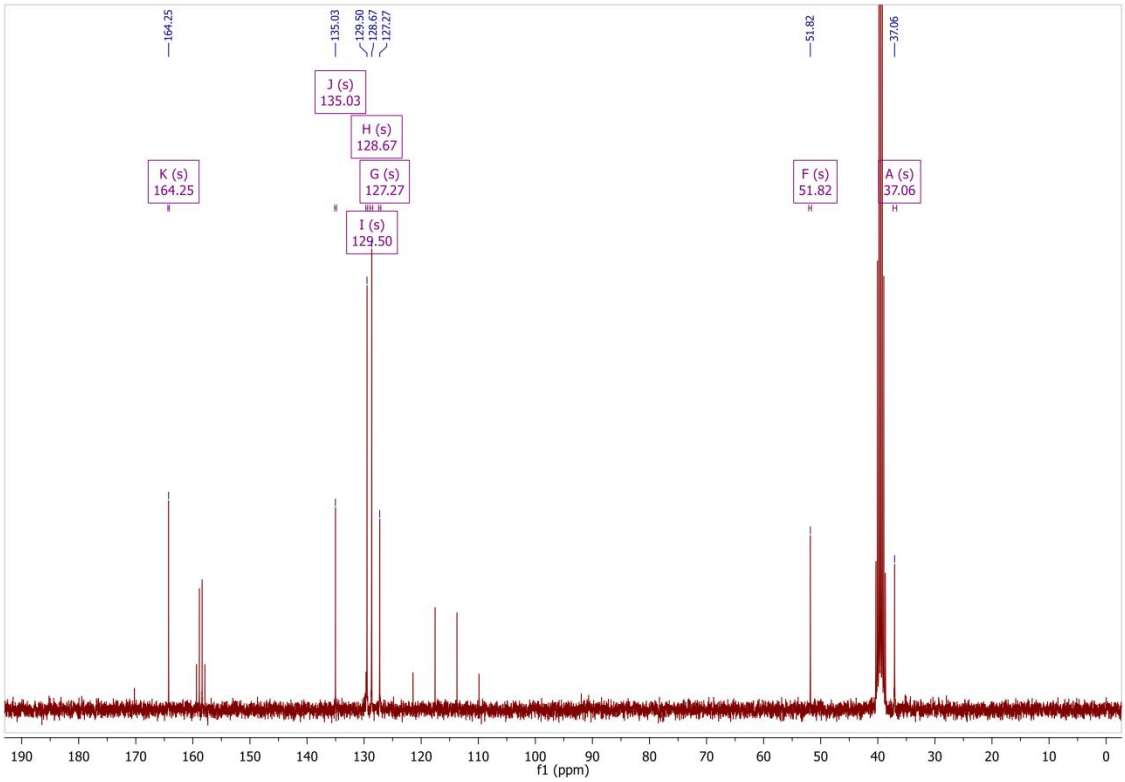
^{1}H NMR spectrum of ProHA (D_2O + 1.8% TFA, 500 MHz).



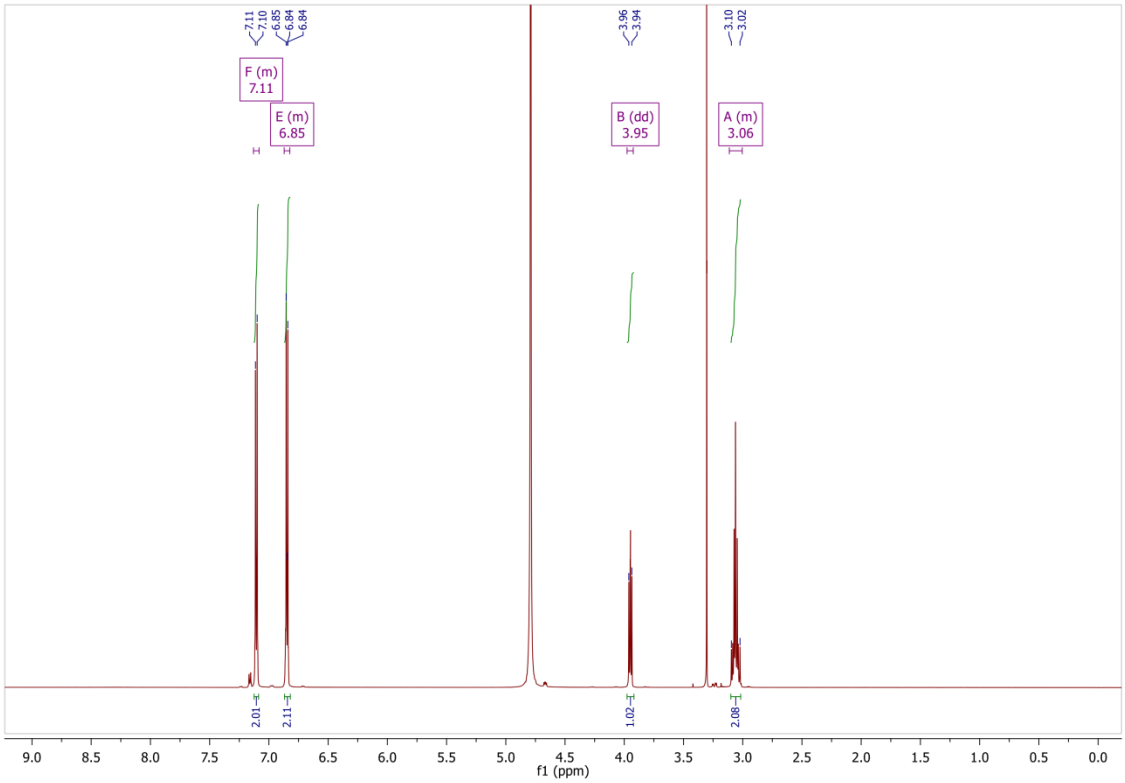
^{13}C NMR spectrum of ProHA (D_2O + 1.8% TFA, 126 MHz).



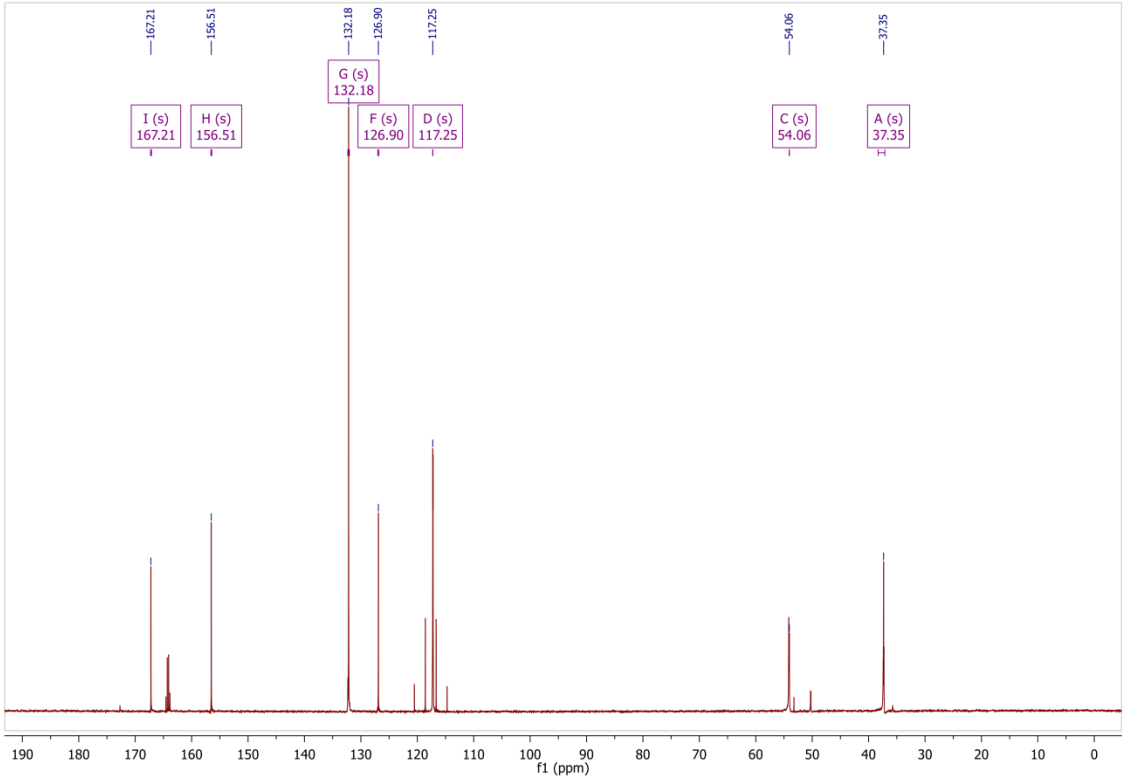
^{1}H NMR spectrum of PheHA (DMSO, 300 MHz).



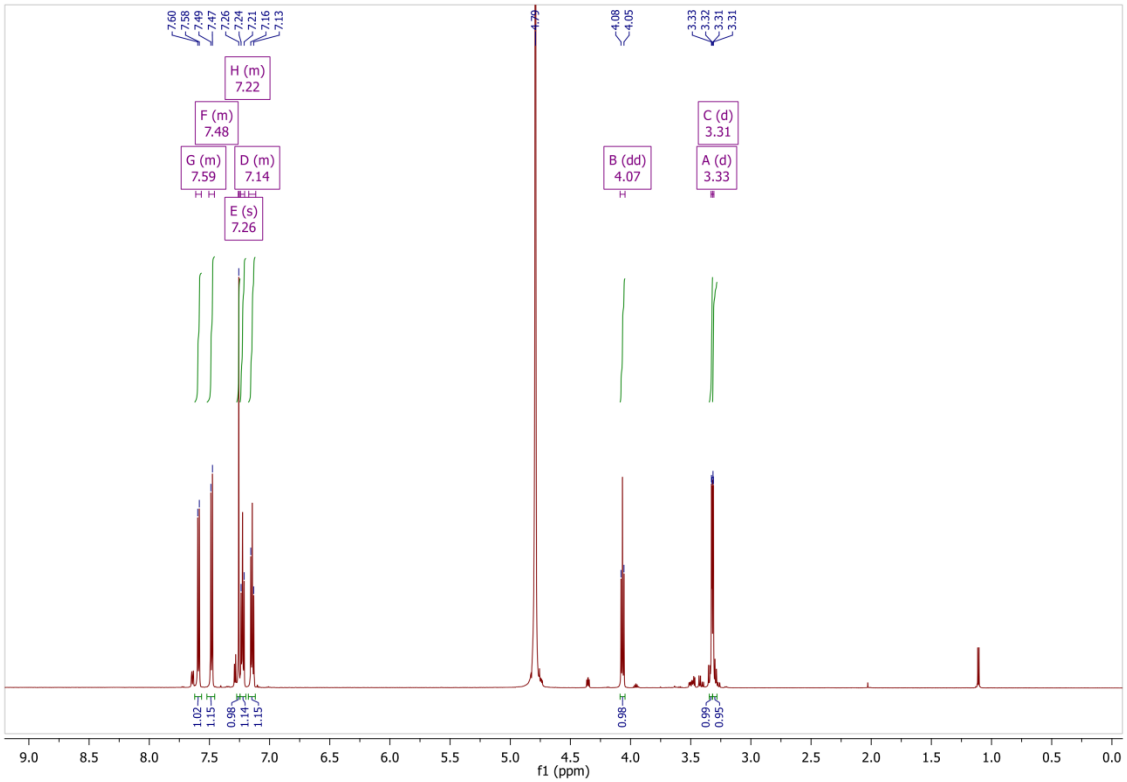
^{13}C NMR spectrum of PheHA (DMSO, 126 MHz).



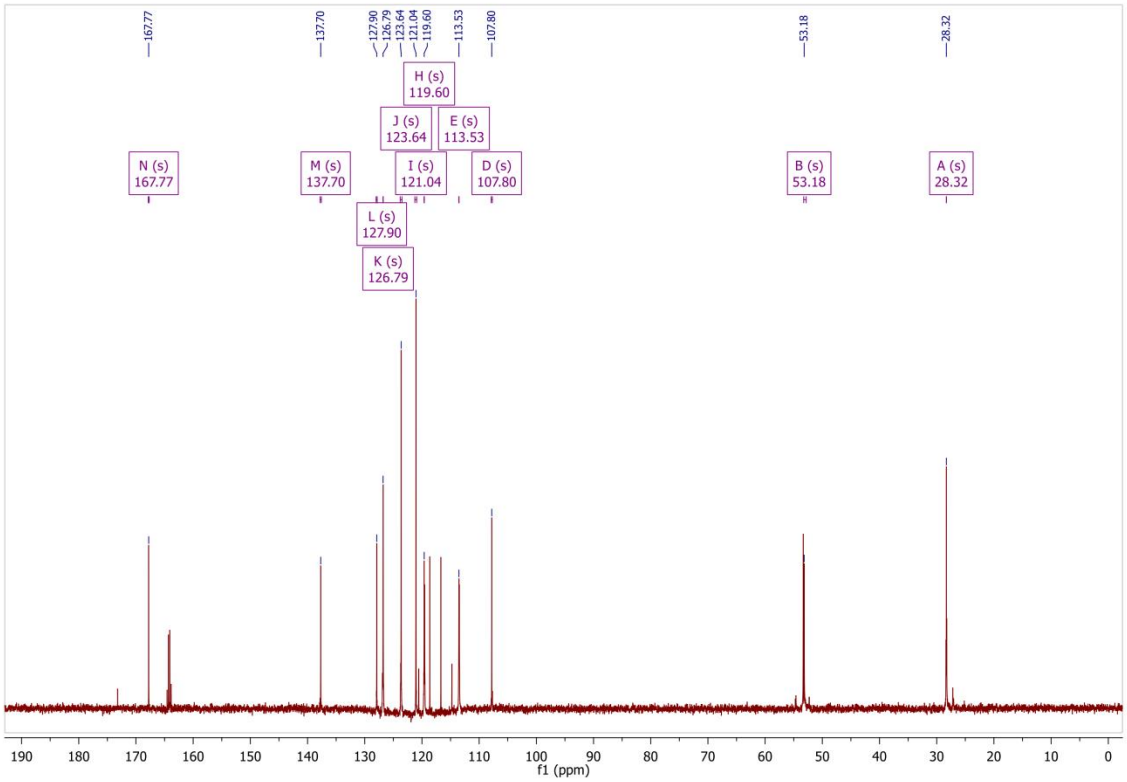
^{1}H NMR spectrum of TyrHA (D_2O + 1.8% TFA, 600 MHz).



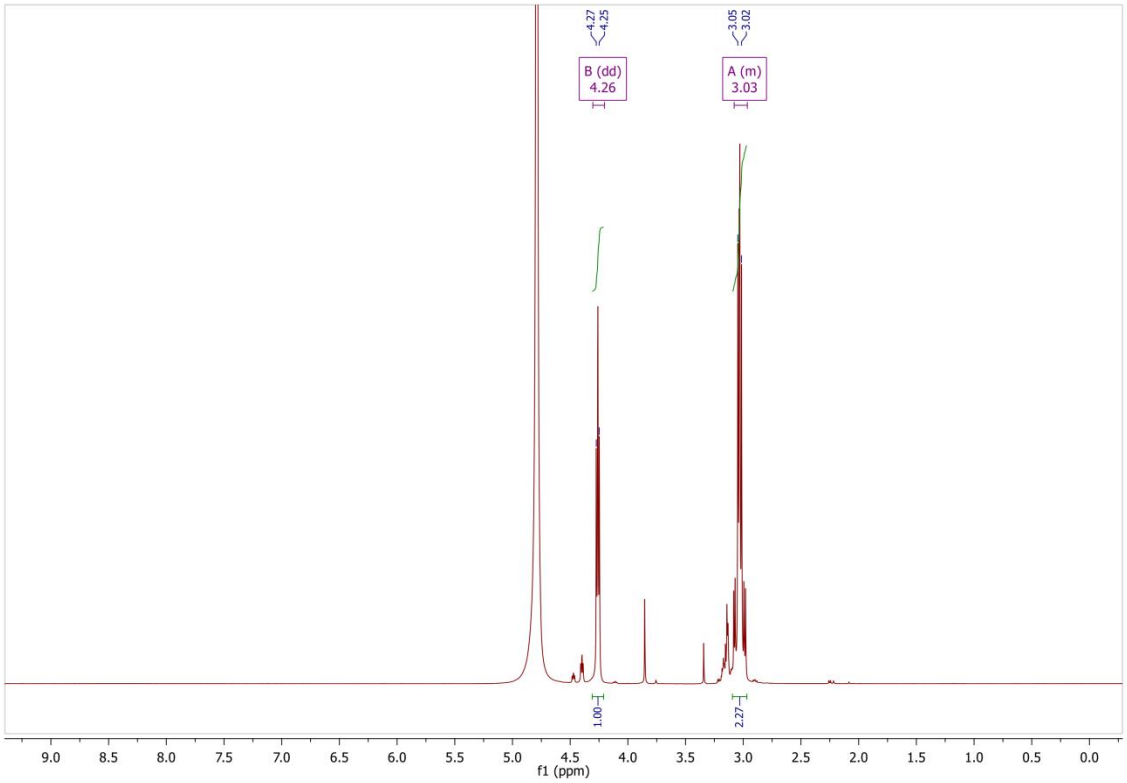
^{13}C NMR spectrum of TyrHA (D_2O + 1.8% TFA, 151 MHz).



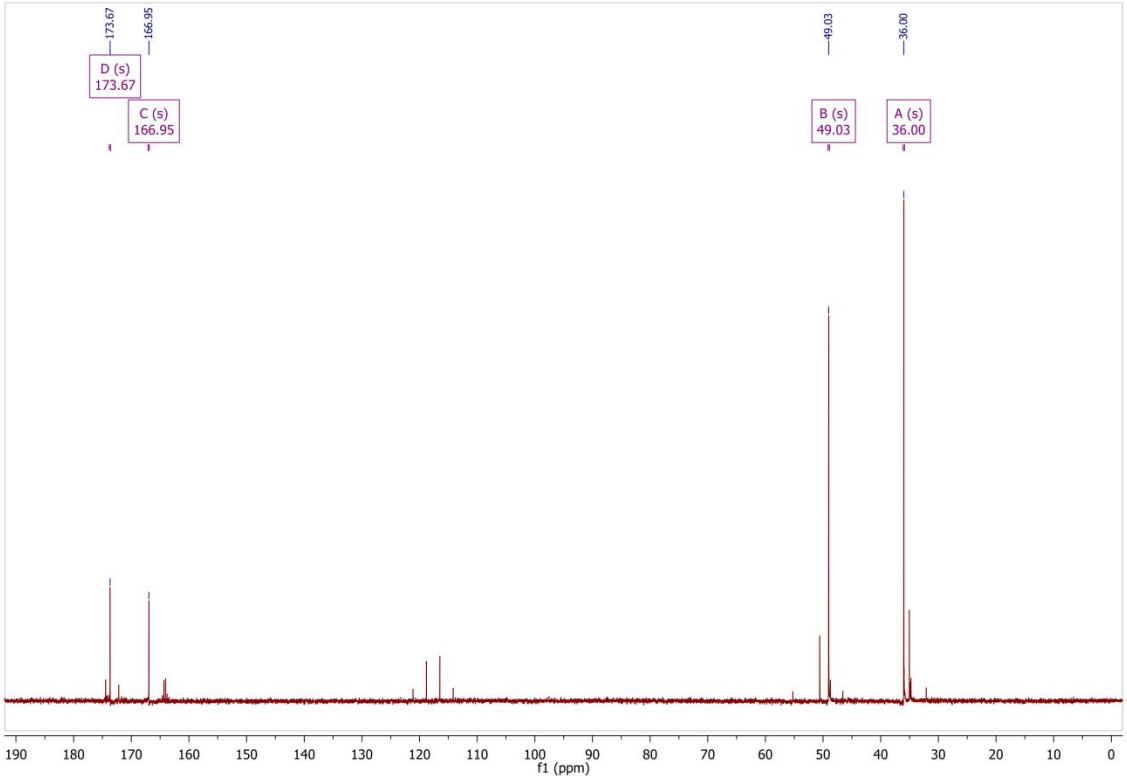
^{1}H NMR spectrum of TrpHA (D_2O + 1.8% TFA, 600 MHz).



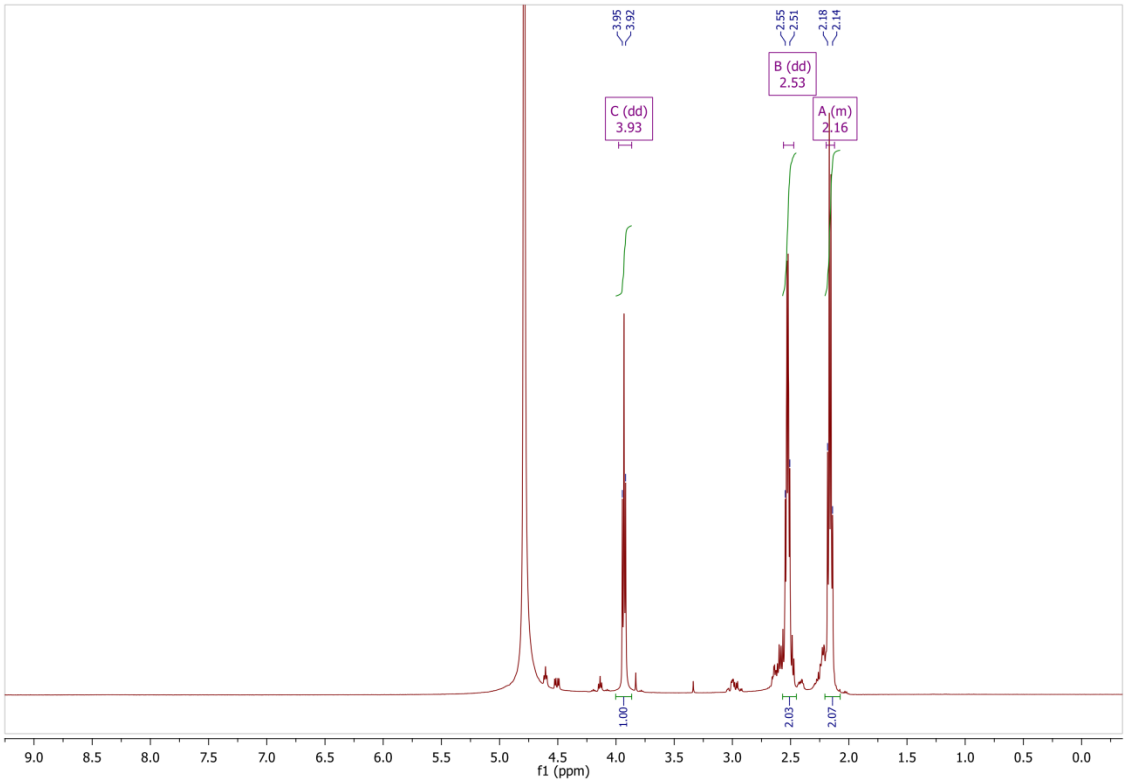
^{13}C NMR spectrum of TrpHA (D_2O + 1.8% TFA, 151 MHz).



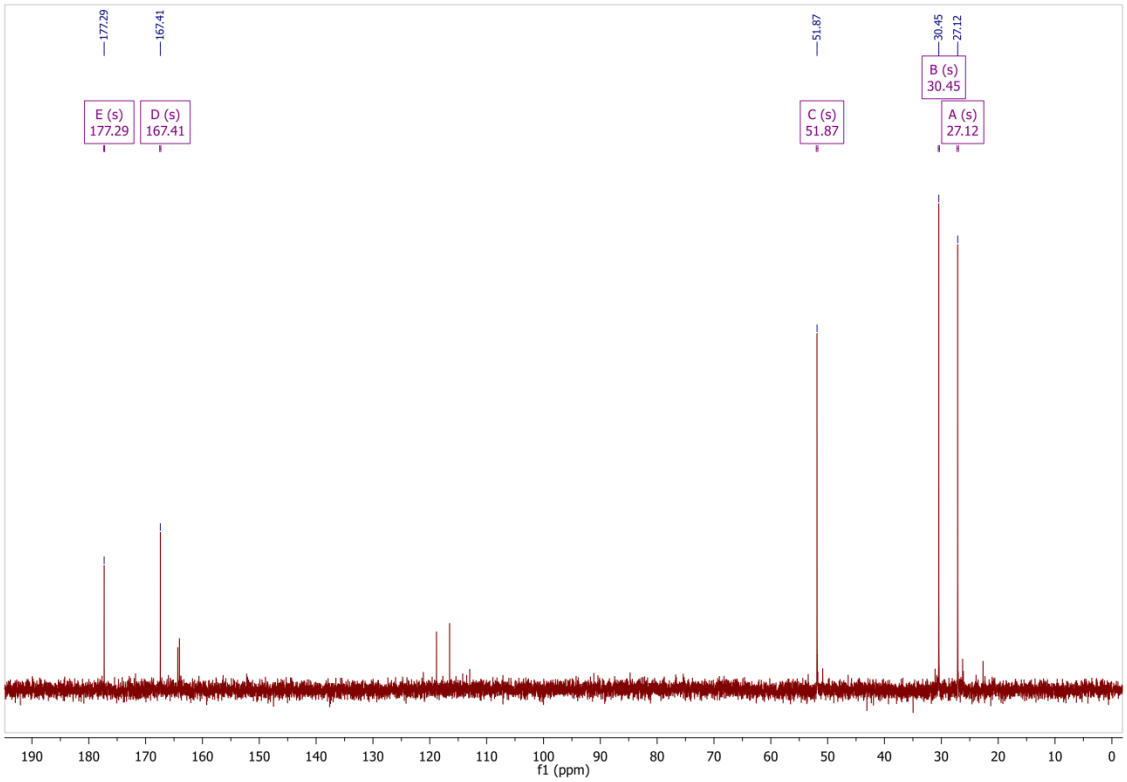
^{1}H NMR spectrum of AspHA (D_2O + 1.8% TFA, 500 MHz).



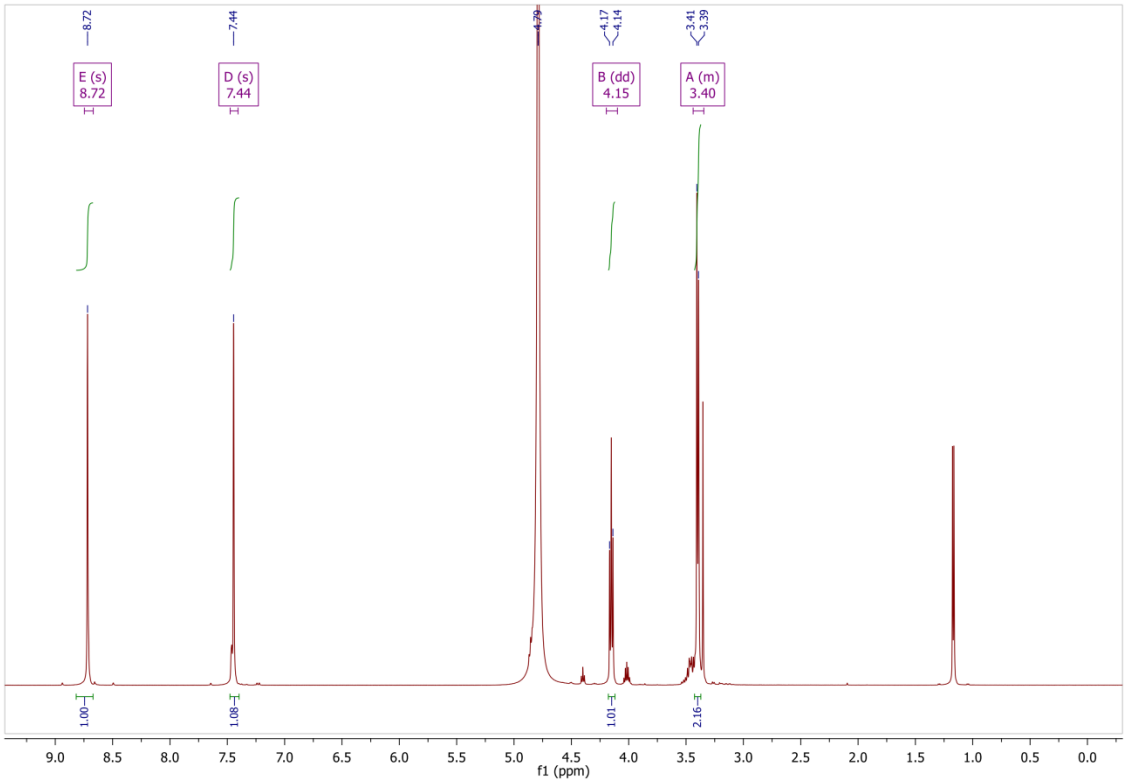
^{13}C NMR spectrum of AspHA (D_2O + 1.8% TFA, 126 MHz).



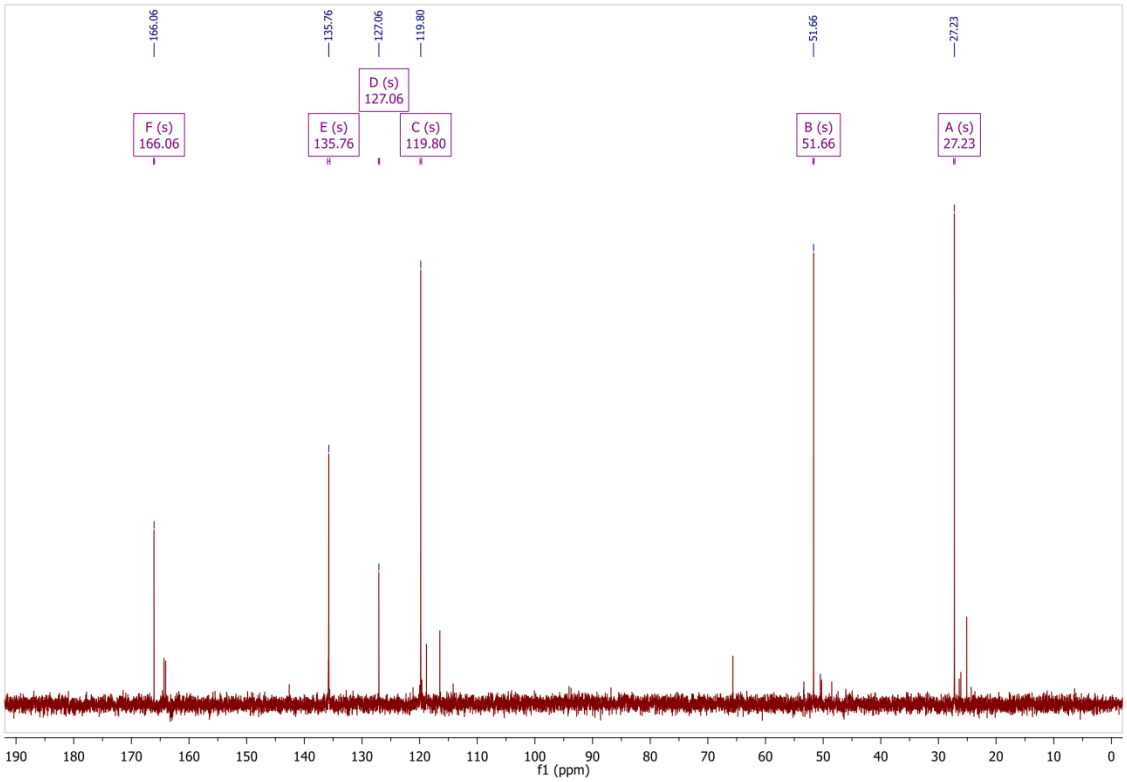
^{1}H NMR spectrum of GluHA (D_2O + 1.8% TFA, 500 MHz).



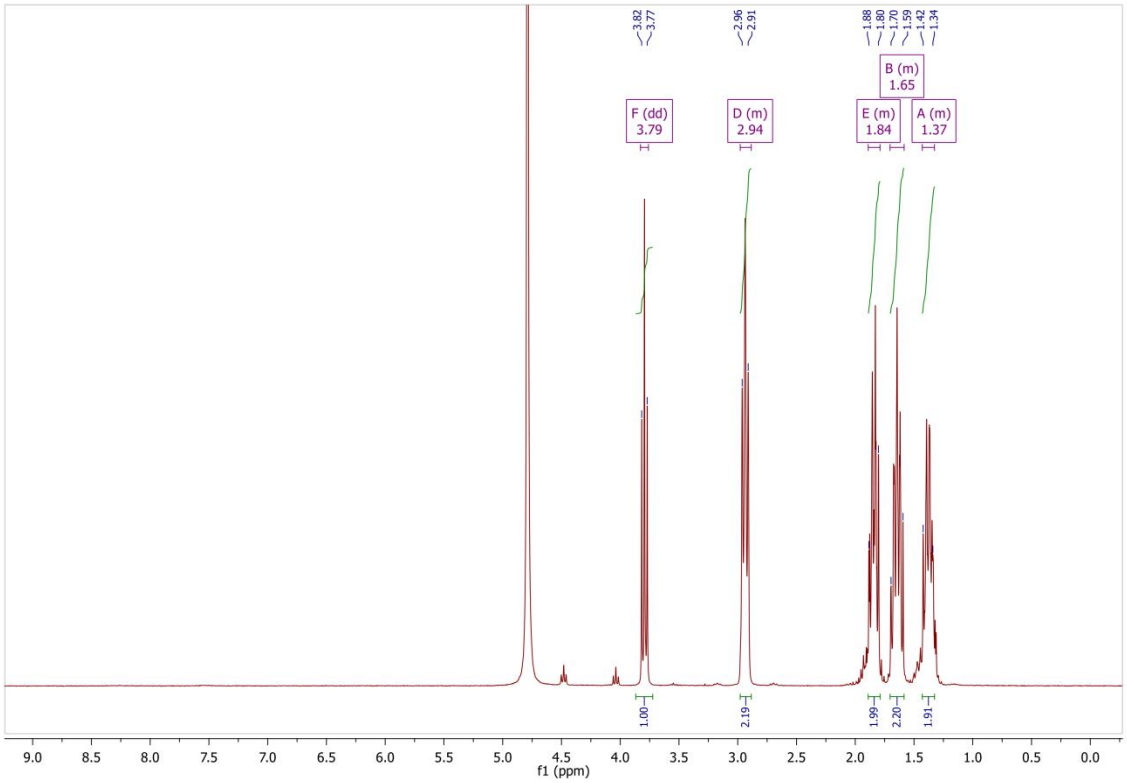
^{13}C NMR spectrum of GluHA (D_2O + 1.8% TFA, 126 MHz).



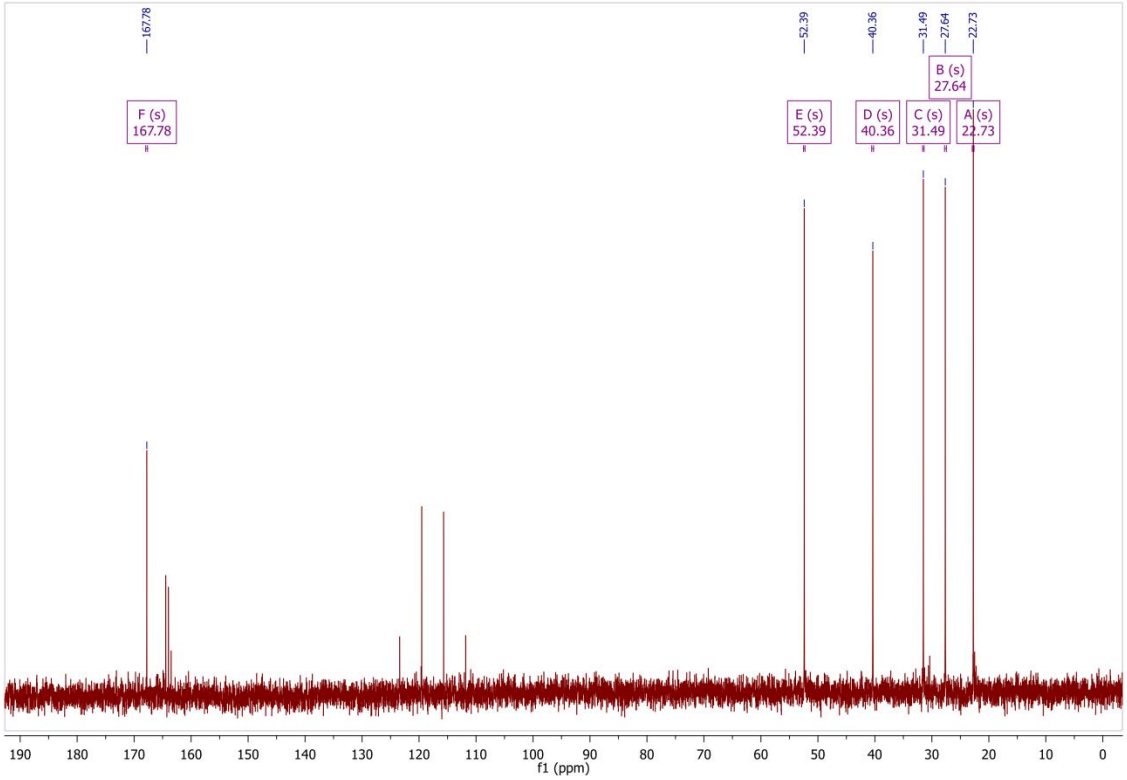
^{1}H NMR spectrum of HisHA (D_2O + 1.8% TFA, 500 MHz).



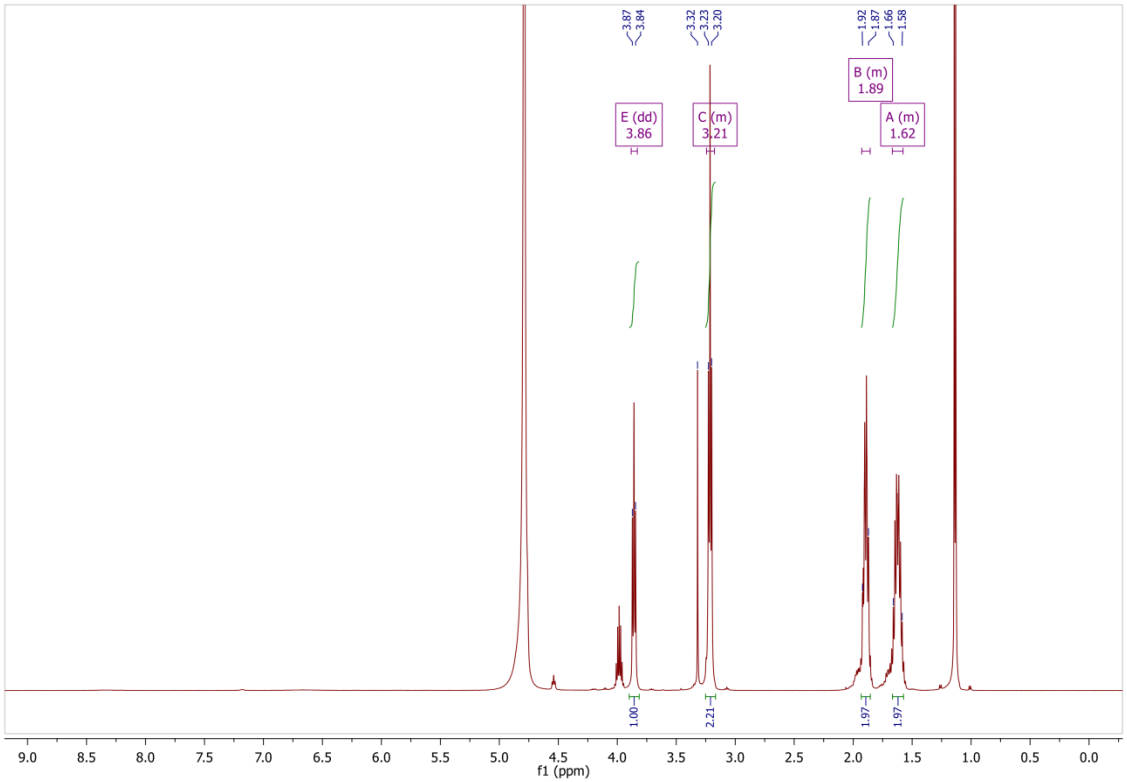
^{13}C NMR spectrum of HisHA (D_2O + 1.8% TFA, 126 MHz).



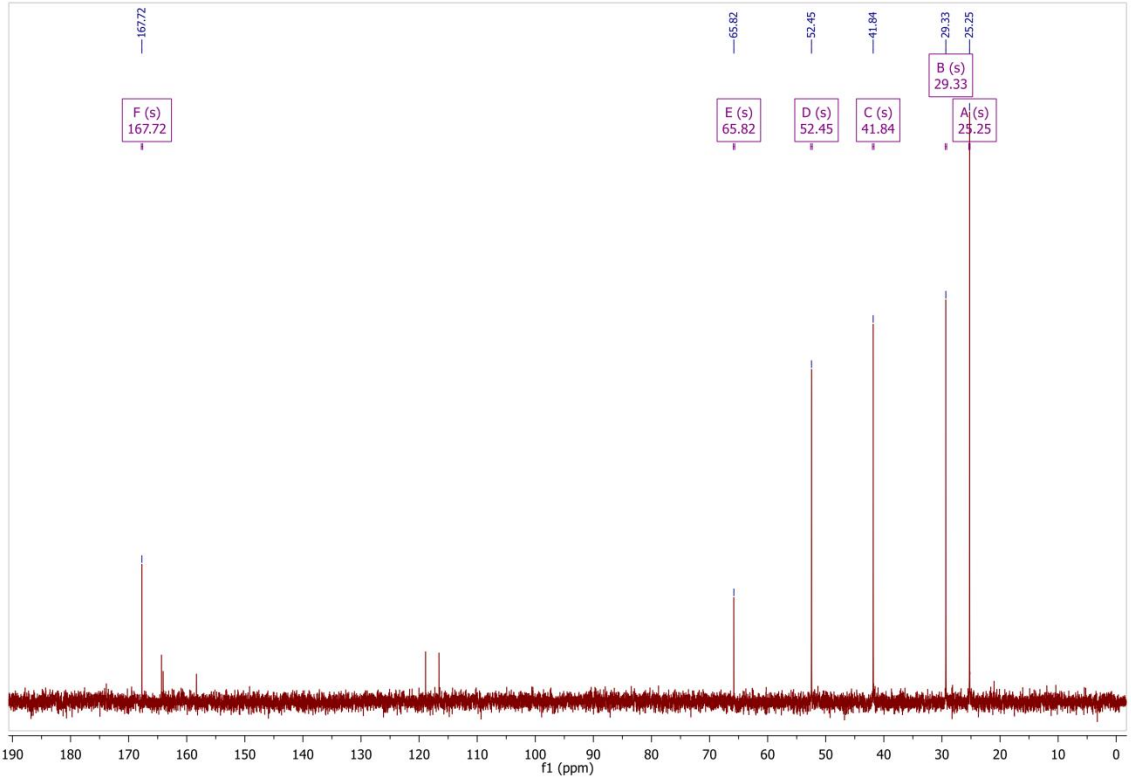
^{1}H NMR spectrum of LysHA (D_2O + 1.8% TFA, 300 MHz).



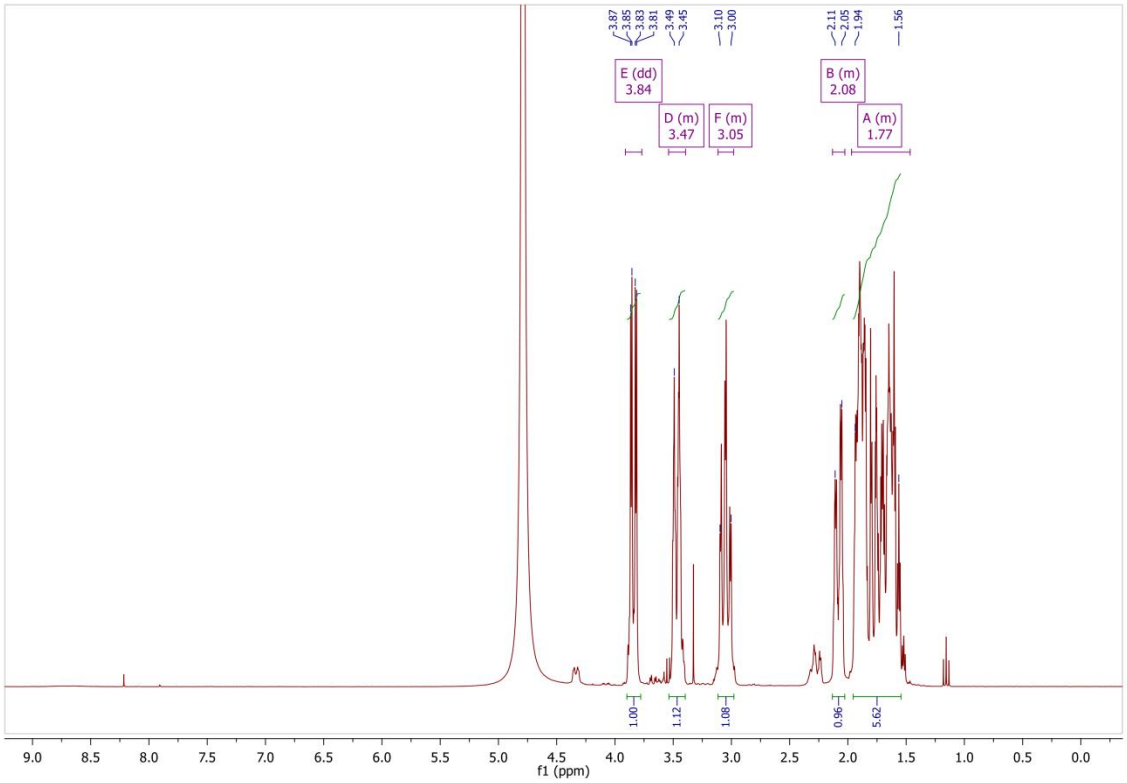
^{13}C NMR spectrum of LysHA (D_2O + 1.8% TFA, 75 MHz).



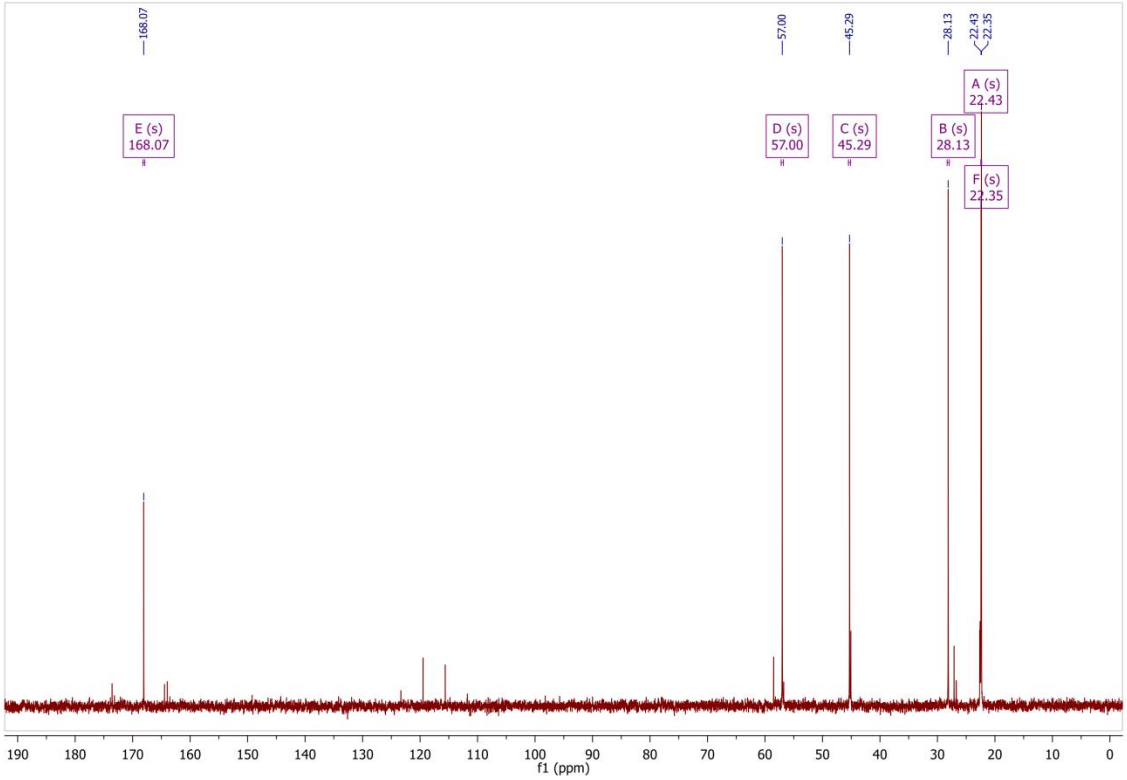
^{1}H NMR spectrum of ArgHA (D_2O + 1.8% TFA, 500 MHz).



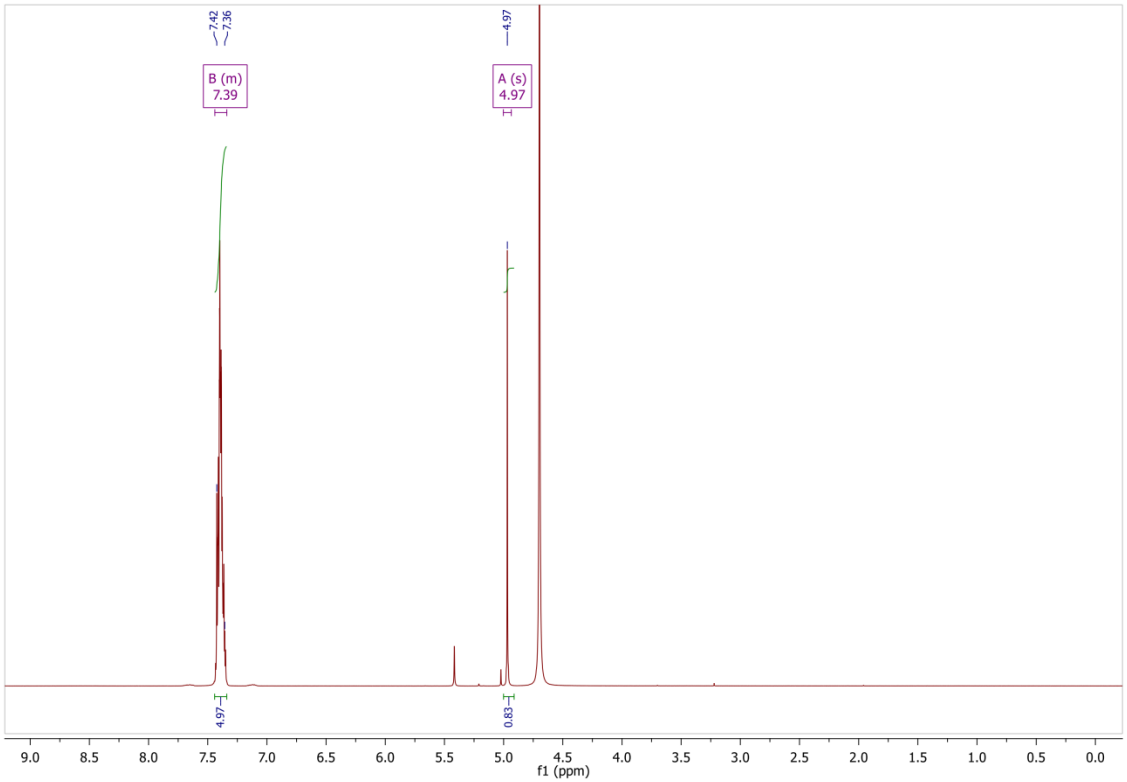
^{13}C NMR spectrum of ArgHA (D_2O + 1.8% TFA, 126 MHz).



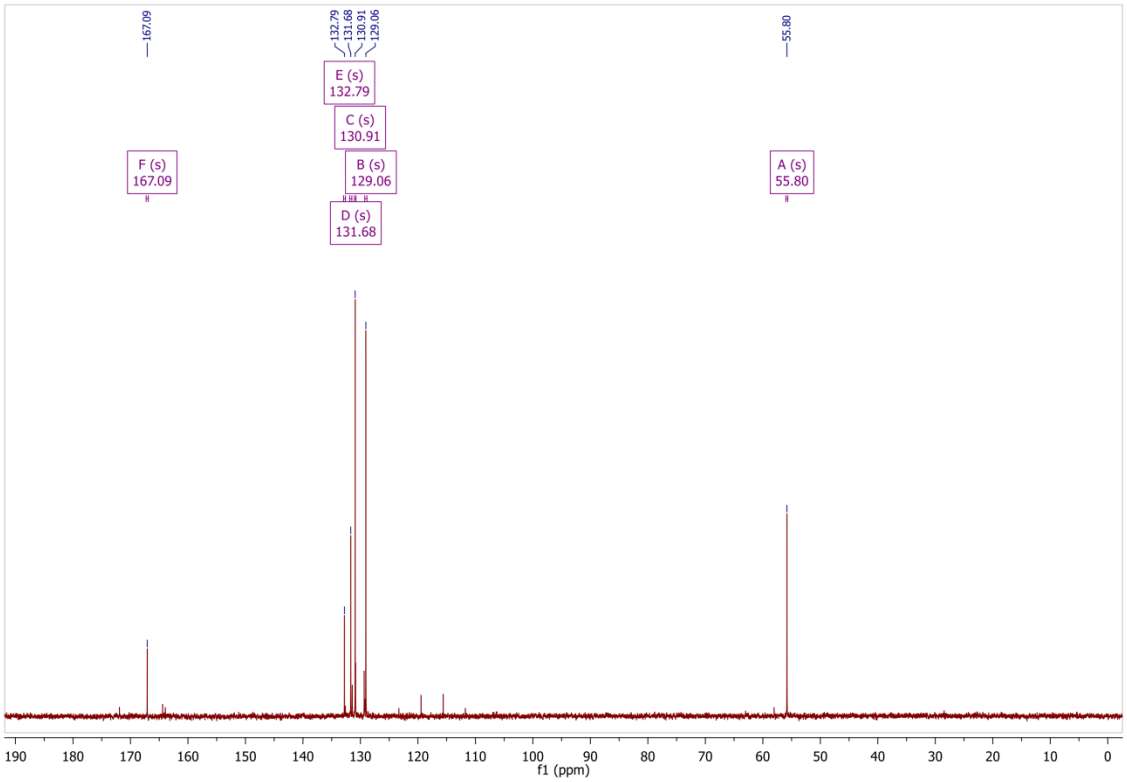
^{1}H NMR spectrum of pipecolic acid HA (D_2O + 1.8% TFA, 300 MHz).



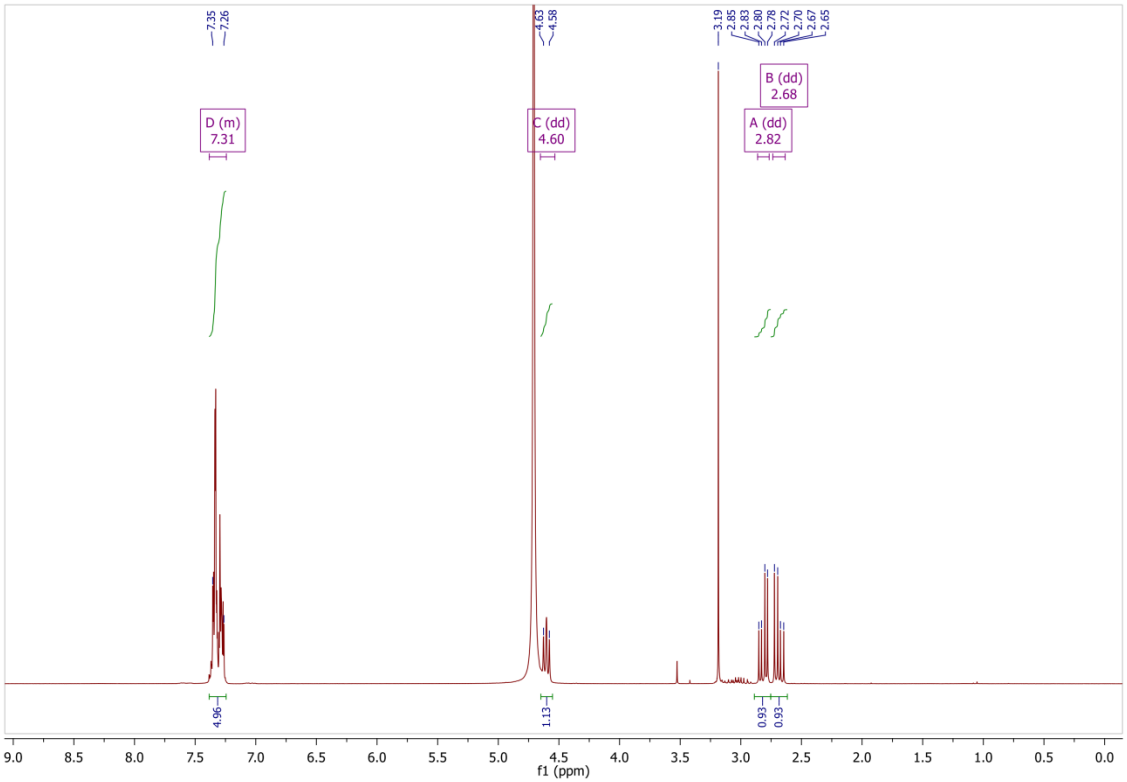
^{13}C NMR spectrum of pipecolic acid HA (D_2O + 1.8% TFA, 75 MHz).



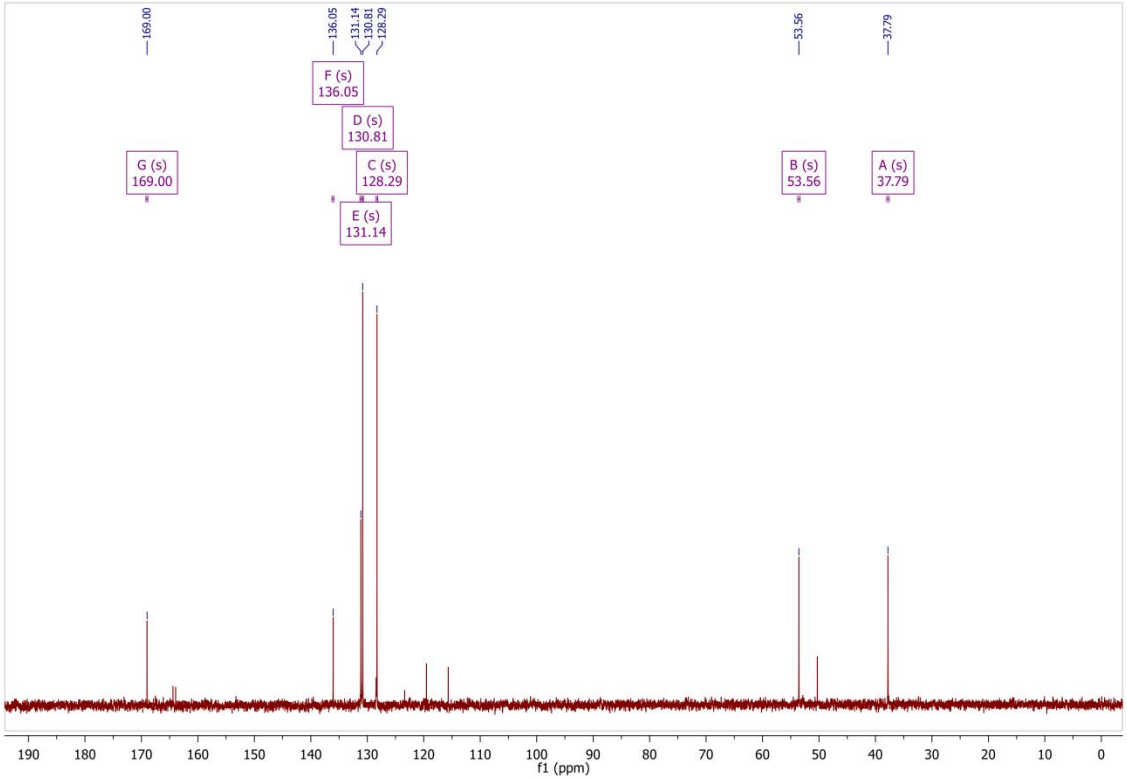
^{1}H NMR spectrum of phenylglycine HA (D_2O + 1.8% TFA, 300 MHz).



^{13}C NMR spectrum of phenylglycine HA (D_2O + 1.8% TFA, 75 MHz).



^{1}H NMR spectrum of β-phenylalanine HA (D_2O + 1.8% TFA, 300 MHz).



^{13}C NMR spectrum of β-phenylalanine HA (D_2O + 1.8% TFA, 75 MHz).

12 Supplementary References

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