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Supporting Information

Context-Dependence of the Reactivity of Cysteine and Lysine Residues

Linus B. Boll and Ronald T. Raines*

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General Experimental Procedures

General Methods

Materials and Reagents

Chemicals were obtained from commercial sources and used without further purification. Rink amide ProTide resin was acquired from CEM corporation (Matthews, NC, USA). 4-Methylpiperidine was from Oakwood Chemical (Estill, SC, USA). H-Cys(Trt)-NH_2, Fmoc-Cys(Trt)-OH, Boc-Lys(Fmoc)-OH, Fmoc-Lys(Boc)-OH, diisopropylethylamine (DIPEA) and HATU were obtained from Chem-Impex International (Wood Dale, IL, USA). Ac-Cys-OH, trifluoroacetic acid (TFA), triisopropylsilane (TIPS), phenol, acetic anhydride, iodomethane, formaldehyde (37 wt% in H_2O), sodium cyanoborohydride, H-Ala-NH_2 hydrochloride, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), tris(2-carboxyethyl)phosphine hydrochloride (TCEP), 1,4-dioxane (anhydrous), *N*-ethylmaleimide, and CH_3CN (anhydrous) were obtained from Sigma Aldrich (Milwaukee, WI, USA). *N*-(Benzoyloxy)succinimide was from TCI (Portland, OR, USA). H_2O indicates "Milli-Q" water prepared by a Milli-Q IQ 7000 Ultrapure Water System from Merck Millipore (Billerica, MA, USA). Procedures were performed at room temperature (~23 °C) unless indicated otherwise.

Purification

Solvents were removed by "concentration under reduced pressure" using a Büchi R210 rotary evaporator at water aspirator pressure (<30 mbar) maintaining the temperature of the water bath at 40 °C. Residual solvent was removed from compounds at high vacuum (<0.1 mbar) using a mechanical belt-drive oil pump. Lyophilization was achieved using a FreeZone 4.5 Liter Freeze Dry System from Labconco (Kansas City, MO, USA). Reverse-phase flash column chromatography was performed on an Isolera One system from Biotage (Uppsala, Sweden) using a Biotage Sfär C18 D Duo (100 Å, 30 µm, 12 g or 100 Å, 30 µm, 30 g) column. H_2O containing 0.1% v/v TFA (solvent A) and CH_3CN containing 0.1% v/v TFA (solvent B) were used as mobile phase. Preparative reverse-phase high-pressure liquid chromatography (RP-HPLC) was performed on a 1260 Infinity II system from Agilent Technologies (Santa Clara, CA, USA). 95%/5% v/v H_2O/CH_3CN containing 0.1% v/v TFA (solvent A) and 95%/5% v/v CH_3CN/H_2O containing 0.1% v/v TFA (solvent B) were used as mobile phase. An XSelect Peptide CSH C18 OBD Prep column (130 Å, 5 µm, 19 mm × 250 mm) from Waters (Wilmslow, UK) was utilized. Analytical RP-HPLC was performed on a 1200 series instrument from Agilent Technologies. H_2O containing 0.1% v/v TFA (solvent A) and CH_3CN containing 0.1% v/v TFA (solvent B) were used as mobile phase. A Microsorb-MV 100-5 C18 column (100 Å, 5 µm, 4.6 mm × 250 mm) from Agilent Technologies was employed. Retention times t_R are defined as the time from injection to elution based on the peak maximum.

Analytical Instrumentation

NMR spectra were recorded on an Avance Neo 500 MHz spectrometer from Bruker (Billerica, MA, USA) at the Department of Chemistry Instrumentation Facility (DCIF) of MIT. ^{13}C spectra were proton-decoupled. ^{1}H chemical shifts are reported in parts per million (ppm, δ scale) referenced to the solvent residual signal (DMSO-d_6: δ 2.50, D_2O: δ 4.79). ^{13}C chemical shifts are reported in parts per million (ppm, δ scale) referenced to the solvent carbon resonance (DMSO-d_6: δ 39.52). For ^{13}C spectra in D_2O, samples were spiked with a small volume of 1,4-dioxane and chemical shifts were referenced to the 1,4-dioxane resonance (δ 67.19).[1] Assignment of signals also relied on the interpretation of COSY and HSQC experiments. For compounds obtained as TFA salts, peaks corresponding to ^{13}C nuclei in trifluoroacetate are not individually reported. High-resolution mass spectra were obtained on an AccuTOF 4G LC-plus spectrometer from JEOL (Peabody, MA, USA) equipped with an ionSense DART source. LC-MS was performed on a 6125B mass spectrometer from Agilent Technologies using electrospray ionization (ESI) attached to a 1260 Infinity LC from Agilent Technologies featuring a Poroshell 120 SB C18 column (2.7 µm, 2.1 mm × 50 mm) from Agilent Technologies. Generally, a gradient of CH_3CN (2–95% v/v) containing formic acid (0.1% v/v) in H_2O containing formic acid (0.1% v/v) over 10 min was used. UV–vis measurements were performed on a Cary 60 UV–vis spectrophotometer from Agilent Technologies. Absorbance measurements at a wavelength of 412 nm were done using disposable methacrylate semi-micro cell cuvettes from Thermo Fisher Scientific (Waltham, MA, USA) with an optical path length of 10 mm. Absorbance at a wavelength of 238 nm was recorded using a Quartz Glass High Performance Type 104B semi-micro cell from Hellma Analytics (Müllheim, Germany) with an optical path length of 10 mm.

Determination of Thiol Concentrations using 5,5'-Dithiobis-(2-nitrobenzoic acid) (DTNB)

100 mM Sodium phosphate buffer containing EDTA (0.1 mM) at pH 7.4 (assay buffer) was prepared and degassed with Ar(g). A solution of DTNB (10 mM) in assay buffer was prepared and degassed with Ar(g). Stock solutions (~40 mM) of compounds **1**–**4** in H_2O were prepared and degassed with Ar(g). In a microcentrifuge tube were mixed 178.0 µL of assay buffer, 20.0 µL of DTNB stock and 2.00 µL of the stock of the respective Cys derivative. A blank solution was prepared by mixing 178.0 µL of assay buffer, 20.0 µL of DTNB stock,

and 2.00 µL of H_2O. For each mixture, 80.0 µL were added to a disposable methacrylate cuvette in 1.200 mL of assay buffer. Absorbance at 412 nm was recorded immediately, and the value for the blank was subtracted. Concentrations were determined using the Beer–Lambert law and a molar absorption coefficient for 5-thio-2-nitrobenzoic acid of 14.15 × 10^3 $M^{-1}cm^{-1}$ at 412 nm.[2] Stock solutions were used for reactions with *N*-ethylmaleimide immediately after determining the concentrations.

Determination of Sulfhydryl pK_a Values

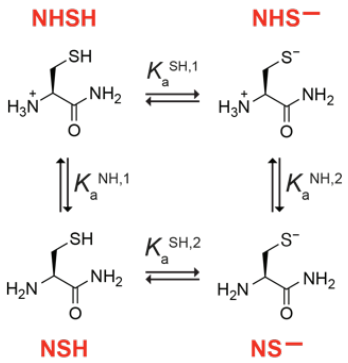
Estimates for pK_a values were obtained by measuring absorbance at 238 nm in dependence of pH at ambient temperature. Buffer stock solutions (100 mM) of KH_2PO_4, K_2HPO_4, and K_3PO_4 were prepared and degassed with Ar(g). A 2 mM stock solution of a Cys derivative in 100 mM KH_2PO_4 was prepared and degassed with Ar(g). Buffer stock solutions were combined in different ratios to give two identical sets of solutions with a volume of 1 mL per solution and varying pH. To the first identical solution, 70.0 µL of 100 mM KH_2PO_4 were added. The mixture was transferred to a quartz glass cuvette and A_{238} was set to zero. To the second identical solution, 70.0 µL of the thiol stock were added and ΔA_{238} was measured in a quartz glass cuvette. The mixture was recovered, and pH was measured on a Fischerbrand accumet XL 500 pH meter from Thermo Fisher Scientific that was freshly calibrated to pH 4.00, pH 7.00, and pH 10.00. The described procedure was repeated for each pair of solutions. For H-Cys-NH_2 and $(Me)_3$-Cys-NH_2 (TFA salt), 2 mM TCEP was added to the buffer and thiol stock solutions to mitigate oxidation across the series of measurements. In absence of 2 mM TCEP, the microscopic sulfhydryl pK_a values found for H-Cys-NH_2 were p$K_a^{SH,1}$ = 7.93(22) and p$K_a^{SH,2}$ = 9.15(4).

To determine the pK_a, ΔA_{238} was fitted against pH by non-linear least-squares fit. For Ac-Cys-OH, Ac-Cys-NH_2 and $(Me)_3$-Cys-NH_2 (TFA salt), the previously reported single titration model was used.[3] For H-Cys-NH_2, a model accounting for the interdependent pK_a values of the sulfhydryl and the α-amino group was used (eq 1). Both models are derived from Beer–Lambert law and the Henderson–Hasselbalch equations for the acid dissociation equilibria.

$$\Delta A_{238} = c_{\text{thiol}} \left(\frac{\varepsilon_{\text{NHSH}} 10^{(\text{p}K_\text{a}^{\text{SH,1}} - \text{pH})} + \varepsilon_{\text{NHS}^-} + \varepsilon_{\text{NSH}} 10^{(\text{p}K_\text{a}^{\text{SH,2}} - \text{p}K_\text{a}^{\text{NH,2}})} + \varepsilon_{\text{NS}^-} 10^{(\text{pH} - \text{p}K_\text{a}^{\text{NH,2}})}}{10^{(\text{p}K_\text{a}^{\text{SH,1}} - \text{pH})} + 1 + 10^{(\text{p}K_\text{a}^{\text{SH,2}} - \text{p}K_\text{a}^{\text{NH,2}})} + 10^{(\text{pH} - \text{p}K_\text{a}^{\text{NH,2}})}} \right) \quad (1)$$

Here, c_{thiol} is the total concentration of thiol. The molar attenuation coefficients $\varepsilon_{\text{NHSH}}$, $\varepsilon_{\text{NHS}^-}$, ε_{NSH} and $\varepsilon_{\text{NS}^-}$ are allocated to the four species as shown in Scheme S1. For the non-linear least-squares fit to this model, it was approximated that the molar attenuation coefficient is not influenced by the protonation state of the amino group; hence, $\varepsilon_{\text{NHSH}} = \varepsilon_{\text{NSH}}$ and $\varepsilon_{\text{NHS}^-} = \varepsilon_{\text{NS}^-}$. It should be noted that a minor blue shift in λ_{max} was observed for the zwitterionic compared to the anionic species.[4] Estimates for the pK_a values were obtained as parameters from the fit. p$K_a^{NH,2}$ was obtained using the pK_a values determined from the fit by application of eq 2.

$$pK_a^{\text{SH,1}} - \text{p}K_\text{a}^{\text{SH,2}} = \text{p}K_\text{a}^{\text{NH,1}} - \text{p}K_\text{a}^{\text{NH,2}} \quad (2)$$



Scheme S1. Species of L-cysteine amide contributing to ΔA_{238} and the acid dissociation constants relevant to the titration model. Identifiers for the species are shown in red.

The obtained fits were plotted as fraction thiolate f^{S-}, calculated using eq 3, against pH, allowing direct comparison among all investigated Cys derivatives.

$$f^{S-} = \frac{\Delta A_{238} - \Delta A_{238}^{\text{min}}}{\Delta A_{238}^{\text{max}} - \Delta A_{238}^{\text{min}}} \quad (3)$$

Determination of Amine Concentrations by qNMR

Stock solutions of Lys derivatives were prepared in D_2O and to part of the solution 1,4-dioxane (anhydrous, 99.8%) was added as a calibrant at a concentration of 80 mM. ^{1}H NMR spectra were recorded using a relaxation delay of 30 s. Concentrations of Lys derivatives were determined by integration of a well-separated signal relative to the 1,4-dioxane signal (s, δ 3.75) at equivalent integral limits.[1] Errors in the obtained concentrations are estimated to be ±2%. Based on the concentrations found by this approach, 10–40 mM stock solutions of Lys derivatives were prepared by diluting with H_2O.

Synthetic Procedures

General Protocols for On-resin Amino Acid Modification

For amino acid derivatives synthesized on resin, Rink amide resin with a maximum loading of 0.59 mmol/g was used. Syntheses were carried out in syringes equipped with a frit and the resin was swollen in CH_2Cl_2 and DMF (10 min each) prior to any of the subsequent steps.

Fmoc Deprotection

To remove Fmoc protecting groups the resin was agitated in 20% v/v 4-methylpiperidine in DMF (3 × 8 min). In between and after cycles, the resin was washed with DMF (3×), CH_2Cl_2 (3×), and DMF (3×).

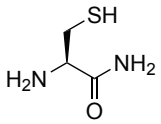
Amino Acid Coupling

Coupling to Rink amide resin was achieved by briefly preactivating the amino acid (3.0 equiv with respect to maximal resin loading) with HATU (2.95 equiv) and DIPEA (8.0 equiv) in DMF (3–5 mL). The mixture was added to the resin followed by agitation for 90 min. The mixture was drained, and the resin was washed using DMF (3×), CH_2Cl_2 (3×) and DMF (3×).

Monitoring of On-resin Procedures

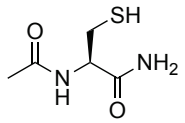
The success of on-resin procedures was monitored by the test cleavage on a small aliquot of resin, which was agitated for 30 min in the same cleavage cocktail (~100 µL) as specified for global cleavage from the resin. The cleavage mixture was evaporated by purging with N_2(g) and a mixture of 1:1 H_2O/CH_3CN or 1:1 H_2O/MeOH (~200 µL) was added. After filtration using a PTFE syringe filter, the solution was analyzed by LC–MS.

L-Cysteine Amide (1)



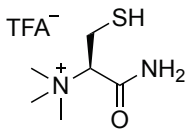
H-Cys(Trt)-NH_2 (156 mg, 0.43 mmol) was dissolved in CH_2Cl_2 (5 mL). A mixture of 80%/10%/5% v/v TFA/TIPS/H_2O, 5% w/v phenol was added. The resulting mixture was stirred for 90 min, during which the initial yellow color from trityl deprotection vanished. Solvents were removed under reduced pressure, and the residue was redissolved in H_2O containing 0.1% v/v TFA. Following filtration, the filtrate was concentrated to about 0.5 mL and subjected to reverse-phase flash column chromatography gradually (0% solvent B for 8 min, 0–100% solvent B over 10 min) to give the TFA salt of H-Cys-NH_2 (**1**) as a white solid (80.0 mg, 0.342 mmol, 80% yield).
^{1}H NMR (500 MHz, D_2O, δ): 4.26 (dd, $^3J_{H,H}$ = 6.0 Hz, $^3J_{H,H}$ = 5.1 Hz, 1H; $C^{\alpha}H$), 3.11 (t, $^3J_{H,H}$ = 5.6 Hz, 2H; $C^{\beta}H_2$).
^{13}C NMR (126 MHz, D_2O, δ): 170.7 (C=O), 54.8 (C^{α}), 25.4 (C^{β}).
HRMS (ESI–TOF): m/z calcd for $C_3H_9N_2OS$ $[M + H]^+$, 121.0441; found, 121.0440.

_N_-Acetyl-L-cysteine Amide (2)



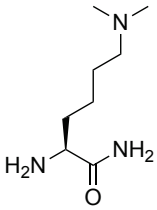
Rink amide resin (687 mg) was swollen and the Fmoc protecting group on the resin was removed as described above. Fmoc-Cys(Trt)-OH was coupled followed by Fmoc deprotection as outlined above. A mixture of acetic anhydride (1.20 mL, 12.7 mmol, 31 equiv with respect to maximal resin loading) and DIPEA (2.14 mL, 12.6 mmol, 31 equiv) in DMF (3 mL) was added, and the resin was agitated with this mixture for 30 min. After washing with DMF (3×), CH_2Cl_2 (3×) and DMF (3×), cleavage was performed using 85%/5%/5% v/v TFA/TIPS/H_2O, 5% w/v phenol (1 × 90 min, 1 × 15 min), and the cleavage mixture was separated by filtration. The mixture was concentrated under reduced pressure, and the residue was taken up into 1:1 CH_3CN/H_2O and filtered. The filtrate was lyophilized, and the residual solid was taken up into H_2O, filtered, and purified by reverse-phase flash column chromatography gradually (C18, 0% solvent B for 5 min, 0–10% solvent B over 5 min, 10–100% solvent B over 8 min) to give Ac-Cys-NH_2 (**2**) as a white powdery solid (27.7 mg, 0.171 mmol, 42% yield).
^{1}H NMR (500 MHz, D_2O, δ): 4.39 (dd, $^3J_{H,H}$ = 6.9 Hz, $^3J_{H,H}$ = 5.2 Hz, 1H; $C^{\alpha}H$), 2.90–2.75 (m, 2H; $C^{\beta}H_2$), 1.98 (s, 3H; CH_3).
^{13}C NMR (126 MHz, D_2O, δ): 175.4 (*C*=O), 175.1 (*C*=O), 56.0 (C^{α}), 25.9 (C^{β}), 22.4 (CH_3).
HRMS (ESI–TOF): *m*/*z* calcd for $C_5H_{11}N_2O_2S$ $[M + H]^+$, 163.0536; found, 163.0530.

_N_,_N_,_N_-Trimethyl-L-cysteine Amide Trifluoroacetate Salt (4)



H-Cys(Trt)-NH_2 (104.8 mg, 0.289 mmol) was suspended in 4:1 v/v H_2O/DMF (5 mL), and 1 M sodium carbonate buffer, pH 9.7 (0.5 mL) was added. Iodomethane (200 µL, 3.21 mmol, 11.1 equiv) was added, and the mixture was agitated at 37 °C overnight. Solvents were removed under reduced pressure by coevaporation with toluene. The residue was taken up into CH_3CN (5 mL) and filtered, and the filtrate was concentrated under reduced pressure. After suspending the residue in CH_2Cl_2 (2.5 mL), a mixture of 80%/10%/5% v/v TFA/TIPS/H_2O, 5% w/v phenol (2.5 mL) was added to give a dark red solution. The solution was stirred for 60 min, over the course of which the color faded. Solvents were removed under reduced pressure, and the residue was taken up into H_2O containing 1% v/v TFA. The mixture was filtered and purified by reverse-phase flash column chromatography gradually (C18, 0% solvent B for 5 min, 0–100% solvent B over 10 min) to give the TFA salt of $(Me)_3$-Cys-NH_2 (**4**) as a white solid (44.2 mg, 0.160 mmol, 55% yield). ^{19}F NMR suggests that the counterion is TFA.
^{1}H NMR (500 MHz, DMSO-d_6, δ): 8.18 (s, 1H; C(=O)N*H*H), 8.06 (s, 1H; C(=O)NH*H*), 3.96 (dd, $^3J_{H,H}$ = 11.4 Hz, $^3J_{H,H}$ = 3.1 Hz, 1H; $C^{\alpha}H$), 3.22 (ddd, $^3J_{H,H}$ = 12.4 Hz, $^3J_{H,H}$ = 7.8 Hz, $^3J_{H,H}$ = 3.1 Hz, 1H; $C^{\beta}H$H), 3.13 (s, 9H; $N(CH_3)_3$), 2.87 (td, $^3J_{H,H}$ = 11.7 Hz, $^3J_{H,H}$ = 9,5 Hz, 1H; C^{β}H*H*), 2.75 (dd, $^3J_{H,H}$ = 9.3 Hz, $^3J_{H,H}$ = 7.8 Hz, 1H; S*H*).
^{13}C NMR (126 MHz, DMSO-d_6, δ): 165.9 (*C*=O), 75.0 (C^{α}), 52.1 ($N(CH_3)_3$), 19.7 (C^{β}).
^{19}F NMR (471 MHz, D_2O, δ): −75.5 (TFA counterion).
HRMS (ESI–TOF): *m*/*z* calcd for $C_6H_{15}N_2OS$ $[M]^+$, 163.0900; found, 163.0893.

N^{ε},N^{ε}-Dimethyl-L-lysine Amide (5)



Rink amide resin (678 mg) was swollen and the Fmoc protecting group on the resin was removed as described above. Boc-Lys(Fmoc)-OH was coupled followed by Fmoc deprotection as outlined above. A mixture of formaldehyde as a 37 wt% solution in H_2O (894 µL, 12.0 mmol, 30.0 equiv with respect to maximal resin loading) in MeOH (3 mL), and DMF (3 mL) was added to the resin followed by agitation overnight. A solution of sodium cyanoborohydride (195 mg, 3.10 mmol, 7.8 equiv) in MeOH (1.5 mL) was added, and agitation was continued for 6 h. The resin was washed with MeOH (3×), DMF (3×), CH_2Cl_2 (3×), and DMF (3×). The imine formation and reduction

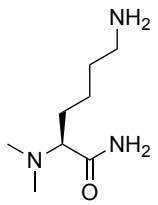
steps were repeated once. Cleavage was performed using 95%/2.5%/2.5% v/v TFA/TIPS/H_2O (1 × 90 min, 1 × 30 min), and the cleavage mixture was separated by filtration. The resin was washed with neat TFA (2×). The combined filtrates were concentrated under reduced pressure, the residue was taken up into 1:1 CH_3CN/H_2O containing TFA (1% v/v) and lyophilized. The residue was taken up into H_2O containing TFA (0.1% v/v) and purified by reverse-phase flash column chromatography gradually (C18, 0% solvent B for 6 min, 0–20% solvent B over 14 min) to give the ditrifluoroacetic acid salt of H-Lys$(Me)_2$-NH_2 (**5**) as a colorless solid (52.0 mg, 0.127 mmol, 32% yield).

^{1}H NMR (500 MHz, D_2O, δ): 4.05 (t, $^3J_{H,H}$ = 6.4 Hz, 1H; $C^{\alpha}H$), 3.16 (m, 2H; $C^{\varepsilon}H_2$), 2.88 (s, 6H; $N(CH_3)_2$), 2.02-1.89 (m, 2H; $C^{\beta}H_2$), 1.85–1.73 (m, 2H; $C^{\delta}H_2$), 1.55–1.42 (m, 2H; $C^{\gamma}H_2$).

^{13}C NMR (126 MHz, D_2O, δ): 172.5 (C=O), 57.8 (C^{ε}), 53.3 (C^{α}), 43.3 (CH_3), 43.2 (CH_3), 30.8 (C^{β}), 24.2 (C^{δ}), 21.7 (C^{γ}).

HRMS (ESI–TOF): *m*/*z* calcd for $C_8H_{20}N_3O$ $[M + H]^+$, 174.1612; found, 174.1607.

N$^{\alpha}$,*N*$^{\alpha}$-Dimethyl-L-lysine Amide (6)



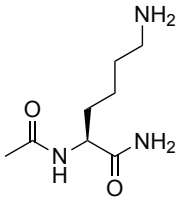
Rink amide resin (680 mg) was swollen and the Fmoc protecting group on the resin was removed as described above. Fmoc-Lys(Boc)-OH was coupled followed by Fmoc deprotection as outlined above. A mixture of formaldehyde as a 37 wt% solution in H_2O (894 µL, 12.0 mmol, 30.0 equiv with respect to maximal resin loading) in MeOH (3 mL), and DMF (3 mL) was added to the resin followed by agitation overnight. A solution of sodium cyanoborohydride (203 mg, 3.23 mmol, 8.1 equiv) in MeOH (1.5 mL) was added, and agitation was continued for 6 h. The resin was washed with MeOH (3×), DMF (3×), CH_2Cl_2 (3×), and DMF (3×). The imine formation and reduction steps were repeated until dimethylation was complete as judged from test cleavage and LC–MS. Cleavage was performed using 95%/2.5%/2.5% v/v TFA/TIPS/H_2O (1 × 90 min, 1 × 30 min) and the cleavage mixture was separated by filtration. The resin was washed extensively using cleavage cocktail (2×) and with neat TFA (2×). The combined filtrates were concentrated under reduced pressure, and the residue was taken up into 1:1 v/v CH_3CN/H_2O containing TFA (1% v/v) and lyophilized. The residue was taken up into H_2O containing TFA (0.1% v/v) and purified by reverse-phase flash column chromatography gradually (C18, 0% solvent B for 9 min, 0–100% solvent B over 8 min) to give the ditrifluoroacetic acid salt of $(Me)_2$-Lys-NH_2 (**6**) as a colorless solid (28.3 mg, 0.071 mmol, 18% yield).

^{1}H NMR (500 MHz, D_2O, δ): 3.87 (dd, $^3J_{H,H}$ = 9.5 Hz, $^3J_{H,H}$ = 4.3 Hz, 1H; $C^{\alpha}H$), 3.02 (t, $^3J_{H,H}$ = 7.7 Hz, 2H; $C^{\varepsilon}H_2$), 2.94 (s, 6H; $N(CH_3)_2$), 2.11–2.01 (m, 1H; $C^{\beta}HH$), 2.00–1.88 (m, 1H; $C^{\beta}HH$), 1.80–1.67 (m, 2H; $C^{\delta}H_2$), 1.53–1.35 (m, 2H; $C^{\gamma}H_2$).

^{13}C NMR (126 MHz, D_2O, δ): 170.8 (C=O), 68.6 (C^{α}), 43.3 (br, CH_3), 41.1 (br, CH_3), 39.6 (C^{ε}), 28.0 (C^{β}), 27.1 (C^{δ}), 21.9 (C^{γ}).

HRMS (ESI–TOF): *m*/*z* calcd for $C_8H_{20}N_3O$ $[M + H]^+$, 174.1612; found, 174.1611.

N$^{\alpha}$-Acetyl-L-lysine Amide (7)



Rink amide resin (970 mg) was swollen and the Fmoc protecting group on the resin was removed as described above. Fmoc-Lys(Boc)-OH was coupled followed by Fmoc deprotection as outlined above. For acetylation, a mixture of acetic anhydride (1.62 mL, 17.1 mmol, 30 equiv with respect to max. resin loading) and DIPEA (2.98 mL, 17.1 mmol, 30 equiv) in DMF (4.5 mL) was agitated with the resin for 25 min. Cleavage was performed using 95%/2.5%/2.5% v/v TFA/TIPS/H_2O (1 × 90 min, 1 × 30 min) and the cleavage mixture was separated by filtration. The mixture was concentrated under reduced pressure. Precipitation from cold diethyl ether gave rise to a cloudy-white dispersion, which was subjected to centrifugation (1500 rpm, 5 min). Diethyl ether was decanted, the residue was taken up into 1/1 v/v CH_3CN/H_2O and lyophilized. The lyophilized solid was taken up into 9/1 v/v H_2O/CH_3CN and purified by RP-HPLC gradually (C18, 0–30% solvent B over 10 min) to afford the TFA salt of Ac-Lys-NH_2 (**7**) as a colorless solid (53.0 mg, 0.176 mmol, 31% yield).

^{1}H NMR (500 MHz, D_2O, δ): 4.24 (dd, $^3J_{H,H}$ = 9.0 Hz, $^3J_{H,H}$ = 5.2 Hz, 1H; $C^{\alpha}H$), 3.00 (t, $^3J_{H,H}$ = 7.6 Hz, 2H; $C^{\varepsilon}H_2$), 2.05 (s, 3H; CH_3), 1.90-1.80 (m, 1H; $C^{\beta}HH$), 1.79-1.63 (m, 3H; $C^{\beta}HH$, $C^{\delta}H_2$), 1.54-1.38 (m, 2H; $C^{\gamma}H_2$).

^{13}C NMR (126 MHz, D_2O, δ): 177.6 (C=O), 175.1 (C=O), 54.0 (C^{α}), 39.8 (C^{ε}), 31.0 (C^{β}), 26.9 (C^{δ}), 22.7 (CH_3), 22.3 (C^{γ}).

^{1}H NMR (500 MHz, DMSO-d_6, δ): 7.92 (s, 1H; N$^{\alpha}H$), 7.76 (s, 2-3H; NH_2/NH_3), 7.34 (s, 1H; C(=O)NHH), 6.98 (s, 1H; C(=O)NHH), 4.14 (m, 1H; C$^{\alpha}H$), 2.76 (m, 2H; C$^{\varepsilon}H_2$), 1.84 (s, 3H; CH_3), 1.61 (m, 1H; C$^{\beta}H$H), 1.48 (m, 3H; C$^{\beta}$HH, C$^{\delta}H_2$), 1.30 (m, 2H; C$^{\gamma}H_2$).
^{13}C NMR (126 MHz, DMSO-d_6, δ): 173.9 (C=O), 169.3 (C=O), 52.1 (C^{α}), 38.7 (C^{ε}), 31.5 (C^{β}), 26.7 (C^{δ}), 22.6 (CH$_3$), 22.4 (C^{γ}).
HRMS (ESI–TOF): *m*/*z* calcd for $C_8H_{18}N_3O_2$ [M + H]$^+$, 188.1405; found, 188.1404.

Competition Experiments

Product Distribution for the Reaction of Cys Derivatives with *N*-Ethylmaleimide

To correct for moisture content of the Cys derivatives, concentrations of the stocks were determined using DTNB as described above. Stocks were prepared in H_2O and degassed with Ar(g). Concentrations were determined just before use. Stock solutions of *N*-ethylmaleimide in assay buffer were used immediately after preparation.

Calibration

To an autosampler vial insert was added PBS, pH 7.4 (volume adjusted to give a final volume of 100 µL). An aliquot of a fresh 5 mM or 20 mM solution of *N*-ethylmaleimide in PBS, pH 7.4 was added to give a concentration of 0.05/0.1/0.2/0.4/0.6/0.8/1 mM with respect to the final volume. Next, an aliquot of a stock solution of a Cys derivatives **1**–**4** was added to produce an excess (≥2-fold) of thiol with respect to *N*-ethylmaleimide. After thorough mixing, mixtures were analyzed by analytical RP-HPLC using an injection volume of 25 µL and a gradient (0% solvent B for 3 min, 0–30% solvent B over 16 min). Absorbance was monitored at 210 nm. The full conversion of *N*-ethylmaleimide was confirmed based on the disappearance of the corresponding peak at t_R = 9.1 min. Summed peak areas for the pair of diastereomers in each calibration mixture were determined by integration. Calibration curves were obtained by linear regression of summed peak areas against concentration forcing 0 intercept and used for the determination of product distributions in competition assays both at pH 7.4 and pH 5.0. **Figures S1**–**S4** show the RP-HPLC traces, summed peak areas against concentration of Michael addition product and fits for each Cys derivatives **1**–**4**.

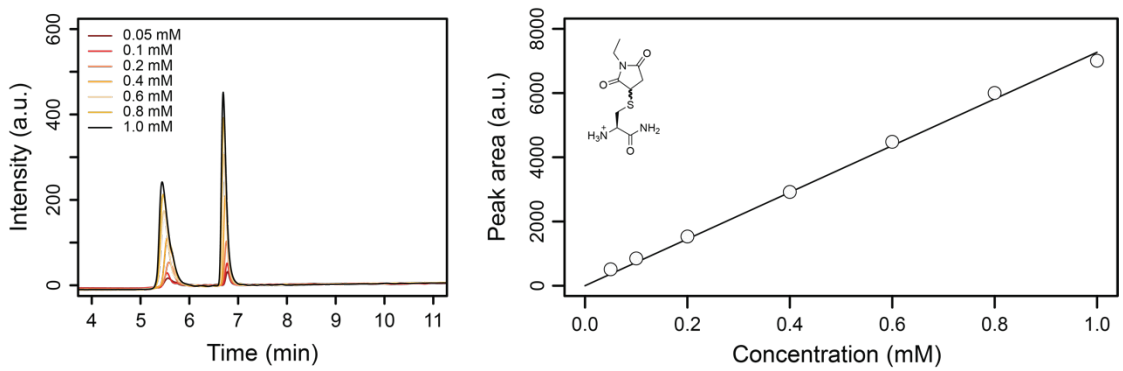


Figure S1. Left: RP-HPLC traces (detection at 210 nm) of calibration mixtures with varying concentrations of Michael addition product of **1** and NEM. Right: Calibration plot for the Michael addition product of **1** and NEM. Sum of the peak areas of both diastereomers at 210 nm against concentration of addition product. Linear regression was performed forcing an intercept of 0. The obtained model was employed to determine product distributions in competition assays.

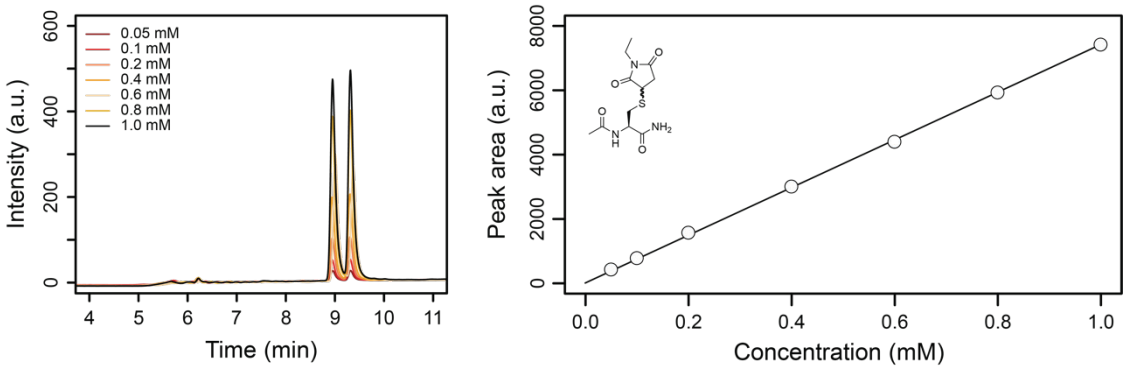
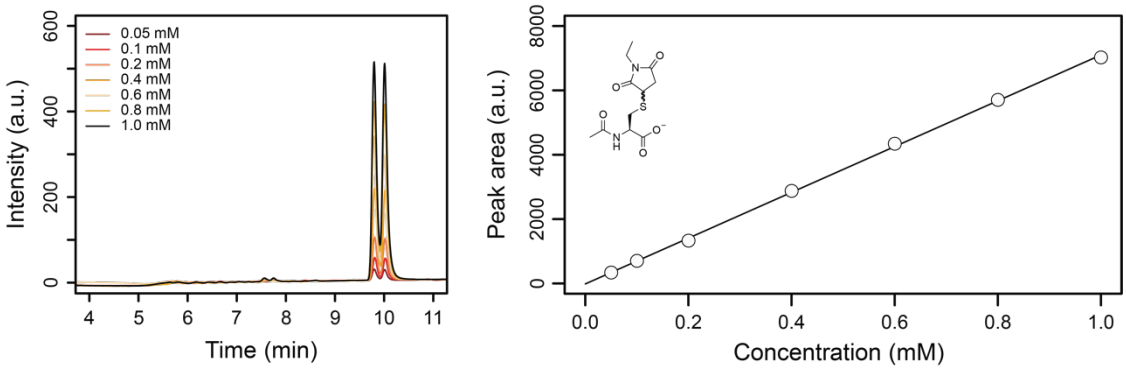


Figure S2. Left: RP-HPLC traces (detection at 210 nm) of calibration mixtures with varying concentrations of Michael addition product of **2** and NEM. Right: Calibration plot for the Michael addition product of **2** and NEM. Sum of the peak areas of both diastereomers at 210 nm against varying concentration of addition product. Linear regression was performed forcing an intercept of 0. The obtained model was employed to determine product distributions in competition assays.

Figure S3. Left: RP-HPLC traces (detection at 210 nm) of calibration mixtures with varying concentrations of Michael addition product of **3** and NEM. Right:



Calibration plot for the Michael addition product of **3** and NEM. Sum of the peak areas of both diastereomers at 210 nm against varying concentration of addition product. Linear regression was performed forcing an intercept of 0. The obtained model was employed to determine product distributions in competition assays.

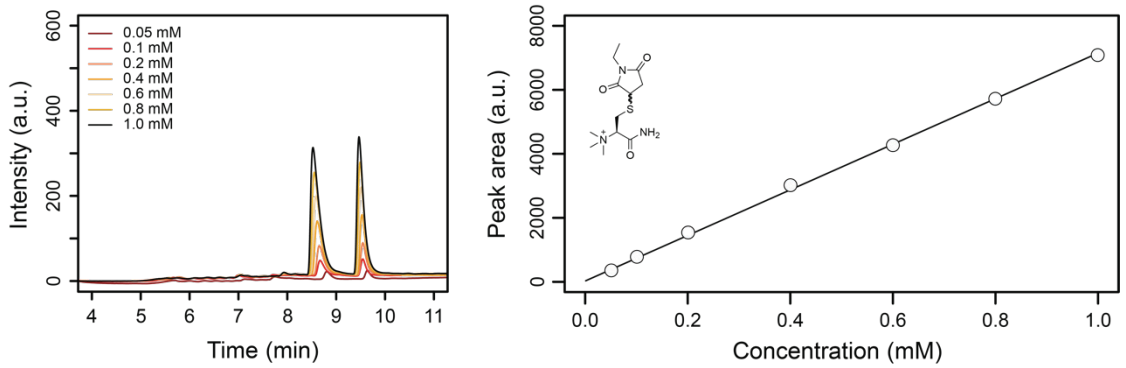


Figure S4. Left: RP-HPLC traces (detection at 210 nm) of calibration mixtures with varying concentrations of Michael addition product of **4** and NEM. Right: Calibration plot for the Michael addition product of **4** and NEM. Sum of the peak areas of both diastereomers at 210 nm against varying concentration of addition product. Linear regression was performed forcing an intercept of 0. The obtained model was employed to determine product distributions in competition assays.

Competition Assays

In an autosampler vial insert was prepared 100 µL of a solution containing 1 mM of Cys derivatives **1**–**3** or **1** and **4** as well as 1 mM *N*-ethylmaleimide in PBS, pH 7.4 by adding buffer, aliquots of stock solutions of the Cys derivatives and 5 µL of a fresh 20 mM stock of *N*-ethylmaleimide in PBS, pH 7.4, in that order. After thorough mixing, the mixture was analyzed by analytical RP-HPLC using an injection volume of 25 µL and a gradient (0% solvent B for 3 min, 0–30% solvent B over 16 min). Absorbance was monitored at 210 nm. Summed peak areas were determined for each set of diastereomers by integration and the product distribution was determined by application of the regressional parameters obtained from calibration. Competition assays at pH 5.0 were set up following the same procedure, except that 30 mM sodium acetate buffer, pH 5.0, was used instead of PBS. Experiments were performed at least in triplicate. Representative RP-HPLC traces for each assay are shown in **Figures S5**–**S7**.

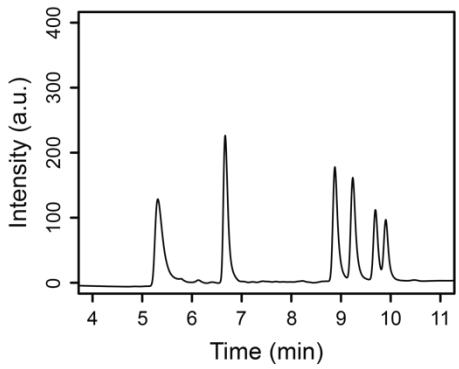


Figure S5. Representative RP-HPLC trace (detection at 210 nm) for the competition assay involving Cys derivatives **1**–**3** in PBS, pH 7.4. The product distribution was obtained by integrating peaks for each set of diastereomers and applying the regressional parameters from calibration (**Figures S1**–**S3**).

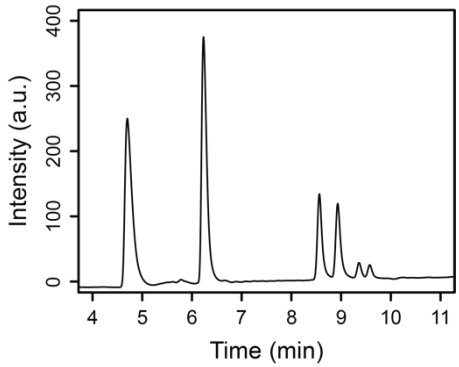


Figure S6. Representative RP-HPLC trace (detection at 210 nm) for the competition assay involving Cys derivatives **1**–**3** in 30 mM sodium acetate buffer, pH 5.0. The product distribution was obtained by integrating peaks for each set of diastereomers and applying the regressional parameters from calibration (**Figures S1**–**S3**).

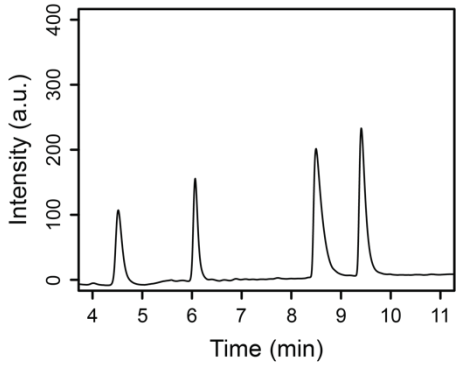


Figure S7. Representative RP-HPLC trace (detection at 210 nm) for the competition assay involving Cys derivatives **1** and **4** in PBS, pH 7.4. The product distribution was obtained by integrating peaks for each set of diastereomers and applying the regressional parameters from calibration (**Figures S1** and **S4**).

For an analysis of the influence of salt concentration on relative reactivity, competition assays involving Cys derivatives **1**–**3** were additionally set in 5 mM sodium acetate buffer, pH 5.0, 300 mM sodium acetate buffer, pH 5.0, and 20 mM sodium phosphate buffer, pH 7.4. Competition assays were set up following the same procedure as outlined above except for experiments in 5 mM sodium acetate buffer, pH 5.0, where readjustment of pH prior to addition of *N*-ethylmaleimide was necessary. Here, a mixture of 5 mM sodium acetate buffer, pH 5.0, (480 µL) and 1 mM of Cys derivatives **1**–**3** with respect to the final volume of 600 µL was prepared. pH was readjusted to 5.0 using 50 mM NaOH. 5 mM sodium acetate buffer, pH 5.0, (volume adjusted to give a final volume of 600 µL) as well as 30 µL of a fresh 20 mM stock of *N*-ethylmaleimide in 5 mM sodium acetate buffer, pH 5.0, were added. After thorough mixing, 100 µL were transferred to an autosampler vial insert and analyzed as outlined above. Experiments were performed at least in triplicate.

Product distributions for each assay were determined by application of the regressional parameters from calibration (**Figures S1**–**S4**) to the peak areas observed upon RP-HPLC analysis of competition experiments and are shown in **Figure S8**. Percentages reflect shares of products with respect to the total amount of Michael addition products.

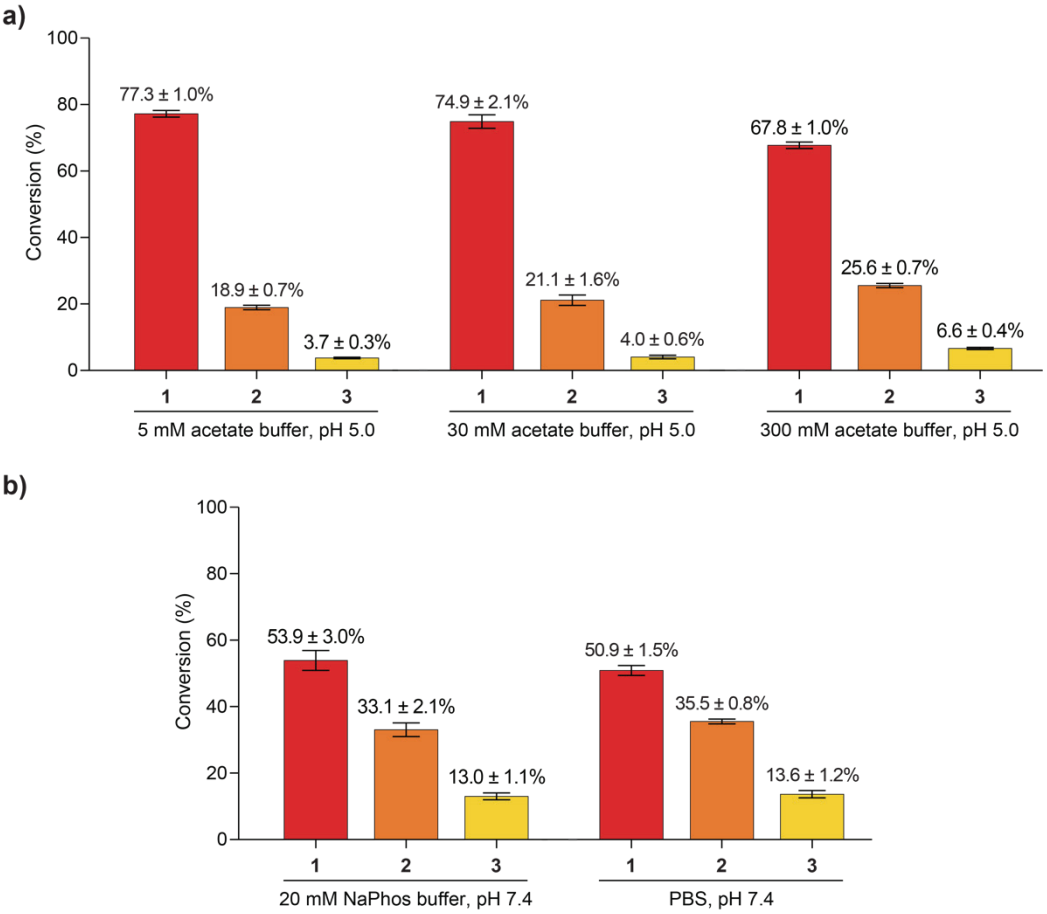


Figure S8. Product distributions determined for each assay by application of the regressional parameters from calibration (**Figures S1**–**S4**) to the peak areas observed upon RP-HPLC analysis of competition experiments. Percentages reflect shares of products with respect to the total amount of Michael addition products. Higher selectivity for the N-terminal position is achieved at low salt concentration. Measurements were performed a) at pH 5.0 with varying concentrations of acetate buffer or b) at pH 7.4 in 20 mM NaPhos buffer or PBS.

Brønsted-type Linear Free-energy Relationship

The obtained product distributions reflect the relative rate constants of addition of the Cys derivatives to NEM. Hence, a Brønsted-type linear free-energy relationship can be applied (eq 5).[5] For this purpose, product distribution percentages observed in PBS, pH 7.4, were normalized to that of the Michael addition product of **3** and NEM, giving relative rate constants $k_{\mathrm{rel}}^{\mathrm{obs}}$ summarized in **Table S1**. As in excellent approximation only the thiolate form contributes to reactivity, thiolate concentration-independent relative rate constants $k_{\mathrm{rel,S-}}$ were determined using eq 4 and are given in **Table S1**.[5-6] Fractions thiolate $f^{\mathrm{S-}}$ were determined from non-linear least-squares fits of ΔA_{238} against pH and are listed in **Table S1**.

$$k_{\mathrm{rel,S-}} = \frac{k_{\mathrm{rel}}^{\mathrm{obs}}}{f^{\mathrm{S-}}} \tag{4}$$

$$\log(k_{\mathrm{rel,S-}}) = C + \beta_{\mathrm{nuc}} \mathrm{p}K_{\mathrm{a}} \tag{5}$$

In eq 5, C is a constant and its value depends on the reaction conditions chosen. β_{nuc} is the Brønsted coefficient. For Cys derivative **1**, $\mathrm{p}K_{\mathrm{a}}^{\mathrm{SH,1}}$ was used in the Brønsted plot. β_{nuc} was obtained by linear regression of $\log(k_{\mathrm{rel,S-}})$ against pK_{a} (eq 5).

Table S1. Values of $k_{\mathrm{rel}}^{\mathrm{obs}}$, $f^{\mathrm{S}-}$ and $k_{\mathrm{rel,S}-}$ used for Brønsted-type analysis. $k_{\mathrm{rel}}^{\mathrm{obs}}$ was obtained by normalization of the product distributions observed in PBS, pH 7.4, to the share of conversion of **3** and NEM. $f^{\mathrm{S}-}$ at pH 7.4 was determined from non-linear least-squares fits of ΔA_{238} against pH. This analysis assumes that the molar extinction coefficient is independent of the protonation state of the amino group. $k_{\mathrm{rel,S}-}$ was determined using eq 4.

Compound	$(Me)_3$-Cys-NH_2 (TFA salt) (**4**)	H-Cys-NH_2 (**1**)	Ac-Cys-NH_2 (**2**)	Ac-Cys-OH (**3**)
$k_{\mathrm{rel}}^{\mathrm{obs}}$	7.3	3.7	2.6	1
$f^{\mathrm{S}-}$ (pH 7.4)	0.621	0.135	0.032	0.009
$k_{\mathrm{rel,S}-}$	11.8	27.6	81.5	111.1

Product Distribution for the Reaction of Lys/Ala Derivatives with *N*-(Benzoyloxy)succinimide

To correct for moisture content of Lys derivatives **5**–**7**, concentrations of the aqueous stocks were determined by qNMR spectroscopy using 1,4-dioxane as calibrant as described above.

Calibration

To an autosampler vial insert was added PBS, pH 7.4 (volume adjusted to give a final volume of 100 µL). An aliquot of a 10–40 mM stock solution of one of compounds **5**–**9** was added to give a concentration of 0.05/0.1/0.2/0.4/0.6/0.8/1 mM with respect to the final volume. Next, 2.5 µL of a 160 mM stock of *N*-(benzoyloxy)succinimide in anhydrous CH_3CN were added to produce an excess (≥4-fold) of NHS ester with respect to the amino acid derivative. Note that an excess of the NHS ester was used due to its hydrolysis as a competing process to amide bond formation. The solution was mixed and incubated overnight. The mixture was analyzed by analytical RP-HPLC using an injection volume of 25 µL and a gradient (10% solvent B for 2 min, 10–15% solvent B over 12 min, 15–50% solvent B over 4 min for calibration involving compounds **5**–**8**; 2.5–15% solvent B over 19 min, 15–50% solvent B over 4 min for calibration involving compound **9**). Absorbance was monitored at 254 nm. Peak areas of the reaction products in each calibration mixture were determined by integration. Calibration curves were obtained by linear regression of peak area against concentration forcing 0 intercept and used for the determination of product distributions in competition assays. **Figures S9**–**S13** show the RP-HPLC traces, peak areas against concentration of amidation product and fits for each Lys derivative **5**–**8** and H-Ala-NH_2 **9**.

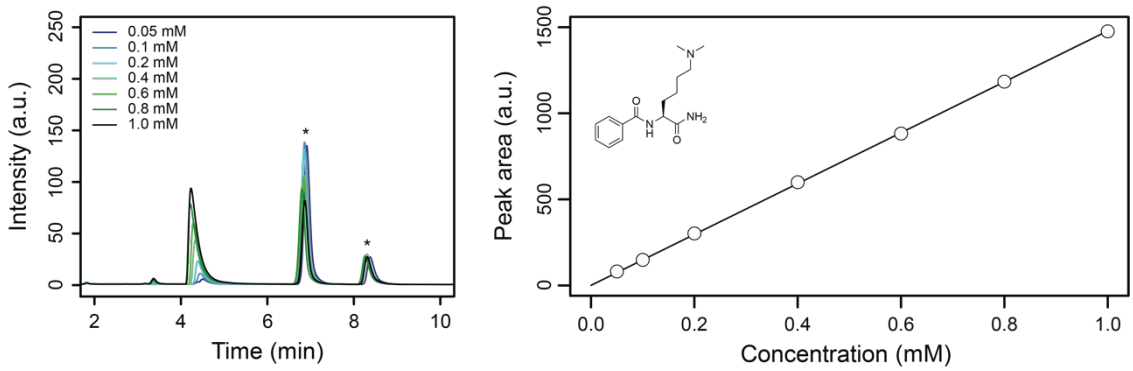


Figure S9. Left: RP-HPLC traces (detection at 254 nm) of calibration mixtures with varying concentrations of amidation product of **5** and *N*-(benzoyloxy)succinimide. Asterisks denote hydrolysis products of the NHS ester. Right: Calibration plot for the amidation product of **5** and *N*-(benzoyloxy)succinimide. Peak area at 254 nm against varying concentration of amidation product. Linear regression was performed forcing an intercept of 0. The obtained model was employed to determine product distributions in competition assays.

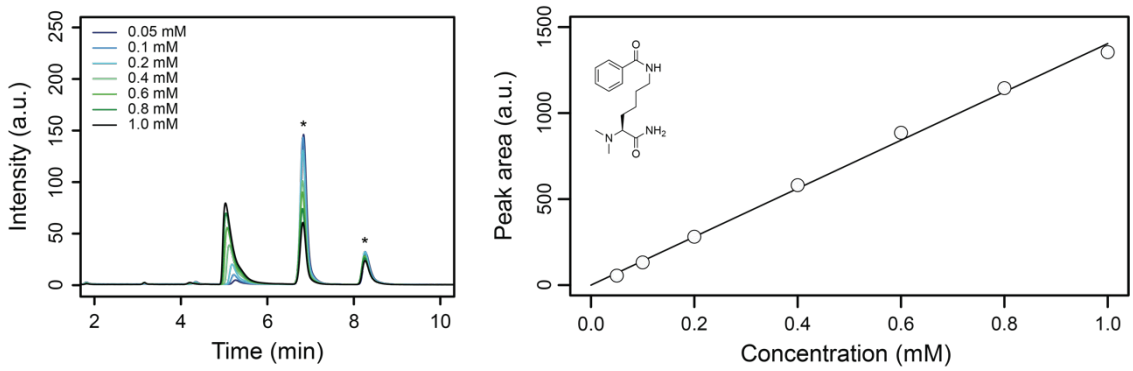


Figure S10. Left: RP-HPLC traces (detection at 254 nm) of calibration mixtures with varying concentrations of amidation product of **6** and *N*-(benzoyloxy)succinimide. Asterisks denote hydrolysis products of the NHS ester. Right: Calibration plot for the amidation product of **6** and *N*-(benzoyloxy)succinimide. Peak area at 254 nm against varying concentration of amidation product. Linear regression was performed forcing an intercept of 0. The obtained model was employed to determine product distributions in competition assays.

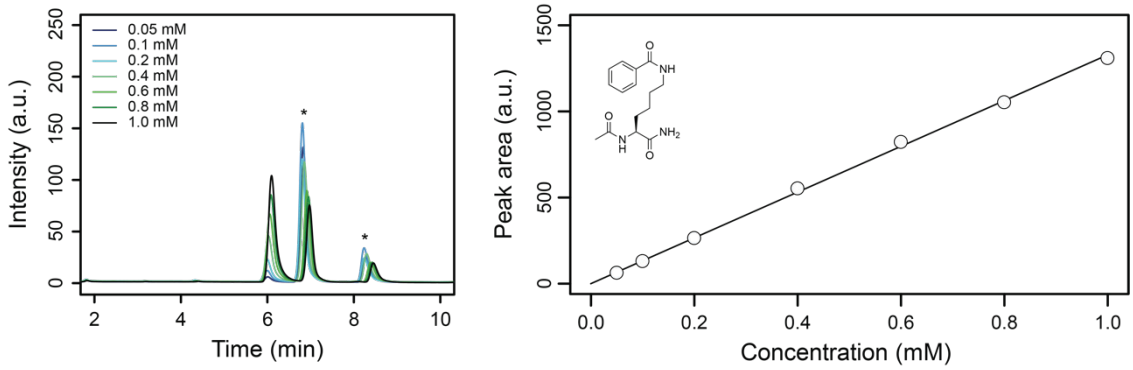


Figure S11. Left: RP-HPLC traces (detection at 254 nm) of calibration mixtures with varying concentrations of amidation product of **7** and *N*-(benzoyloxy)succinimide. Asterisks denote hydrolysis products of the NHS ester. Right: Calibration plot for the amidation product of **7** and *N*-(benzoyloxy)succinimide. Peak area at 254 nm against varying concentration of amidation product. Linear regression was performed forcing an intercept of 0. The obtained model was employed to determine product distributions in competition assays.

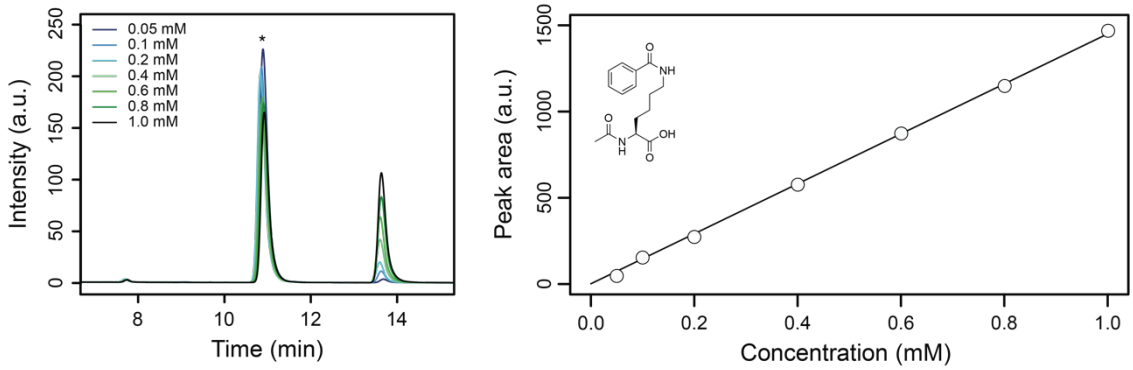


Figure S12. Left: RP-HPLC traces (detection at 254 nm) of calibration mixtures with varying concentrations of amidation product of **8** and *N*-(benzoyloxy)succinimide. Asterisk denotes hydrolysis product of the NHS ester. Right: Calibration plot for the amidation product of **8** and *N*-(benzoyloxy)succinimide. Peak area at 254 nm against varying concentration of amidation product. Linear regression was performed forcing an intercept of 0. The obtained model was employed to determine product distributions in competition assays.

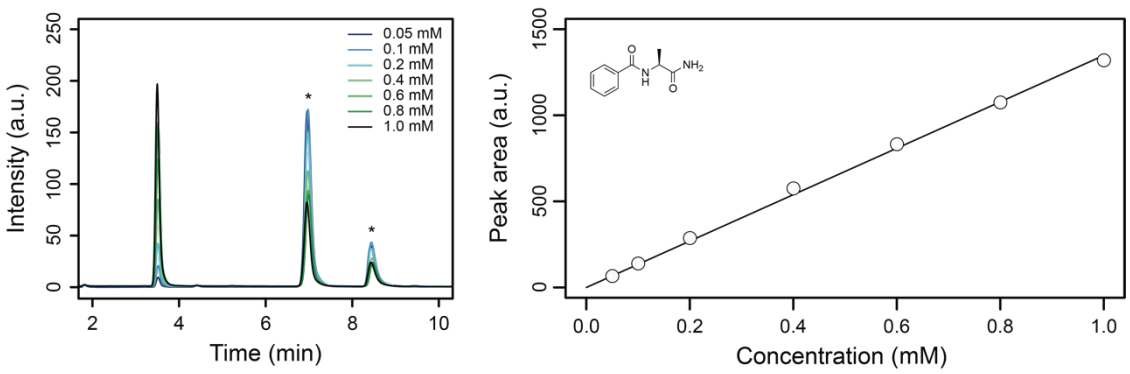


Figure S13. Left: RP-HPLC traces (detection at 254 nm) of calibration mixtures with varying concentrations of amidation product of **9** and *N*-(benzoyloxy)succinimide. Asterisks denote hydrolysis products of the NHS ester. Right: Calibration plot for the amidation product of **9** and *N*-(benzoyloxy)succinimide. Peak area at 254 nm against varying concentration of amidation product. Linear regression was performed forcing an intercept of 0. The obtained model was employed to determine product distributions in competition assays.

Competition Assays

In an autosampler vial insert was prepared 100 µL of a solution containing 1 mM of Lys derivates **5**–**7** or **6**, **7**, and **9** as well as 1 mM of *N*-(benzoyloxy)succinimide in PBS, pH 7.4, by adding buffer, aliquots of stock solutions of the amino acid derivatives, and 2.5 µL of a 40 mM stock of *N*-(benzoyloxy)succinimide in anhydrous CH_3CN, in that order. The solution was mixed and incubated overnight. The mixture was analyzed by analytical RP-HPLC using an injection volume of 25 µL and a gradient (10% solvent B for 2 min, 10–15% solvent B over 12 min, 15–50% solvent B over 4 min). Absorbance was monitored at 254 nm. Peak areas were determined for each product by integration and the product distribution was determined by application of the regressional parameters obtained from calibration. Competition assays involving Lys derivatives **6**–**8** were set up following the same procedure but to improve separation of the products resulting from labelling with *N*-(benzoyloxy)succinimide a different RP-HPLC gradient was used. Mixtures were analyzed by analytical RP-HPLC using an injection volume of 25 µL and a gradient (2.5–15% solvent B over 19 min, 15–50% solvent B over 4 min). Absorbance was monitored at 254 nm. Product distributions were determined as described above. Experiments were performed at least in triplicate. Representative RP-HPLC traces for each assay are shown in **Figures S14**–**S16**.

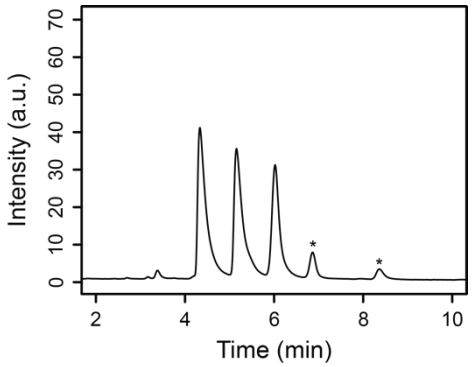


Figure S14. Representative RP-HPLC trace (detection at 254 nm) for the competition assay involving Lys derivatives **5**–**7** in PBS, pH 7.4. The product distribution was obtained by integrating peaks for each set of diastereomers and applying the regressional parameters from calibration (**Figures S9**–**S11**). Asterisks denote hydrolysis products of the NHS ester.

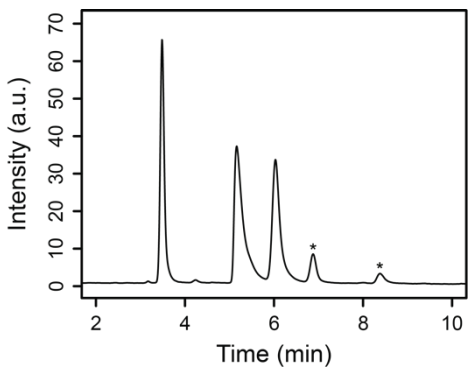


Figure S15. Representative RP-HPLC trace (detection at 254 nm) for the competition assay involving Lys derivatives **6** and **7** as well as H-Ala-NH_2 **9** in PBS, pH 7.4. The product distribution was obtained by integrating peaks for each set of diastereomers and applying the regressional parameters from calibration (**Figures S10**, **S11** and **S13**). Asterisks denote hydrolysis products of the NHS ester.

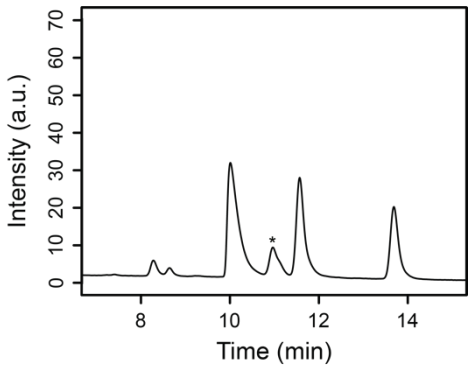


Figure S16. Representative RP-HPLC trace (detection at 254 nm) for the competition assay involving Lys derivatives **6**–**8** in PBS, pH 7.4. The product distribution was obtained by integrating peaks for each set of diastereomers and applying the regressional parameters from calibration (**Figures S10**–**S12**). Asterisks denote hydrolysis products of the NHS ester.

Hydrolysis of N-(Benzoyloxy)succinimide

To identify the byproducts of amine labelling with *N*-(benzoyloxy)succinimide resulting from hydrolysis of the NHS ester, *N*-(benzoyloxy)succinimide was incubated overnight in PBS, pH 7.4, in absence of amines. The mixture was analyzed by analytical RP-HPLC using a gradient (10% solvent B for 2 min, 10–15% solvent B over 12 min, 15–50% solvent B over 4 min) (**Figure S17a**). Absorbance was monitored at 254 nm. Additionally, the mixture was analyzed by LC-MS (detection at 250 nm) using the method described above (**Figure S17b**).

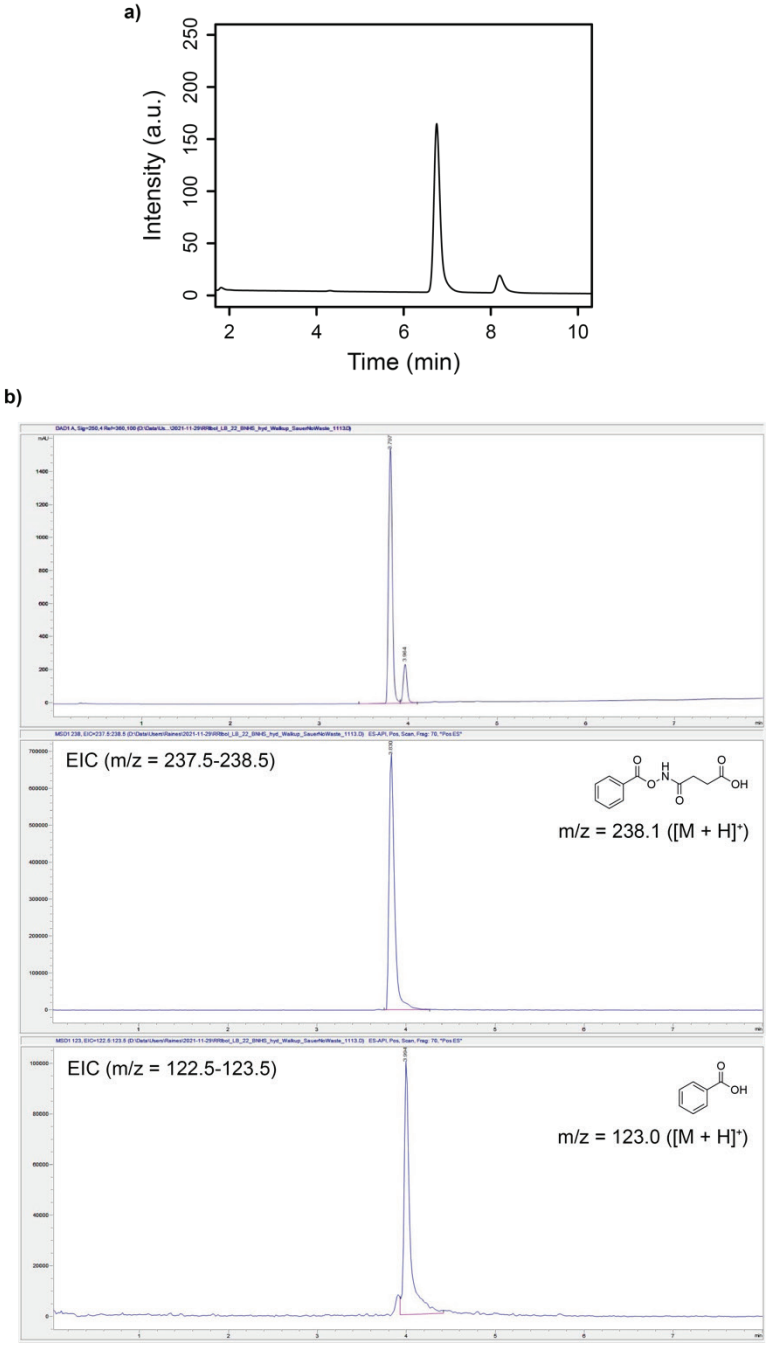
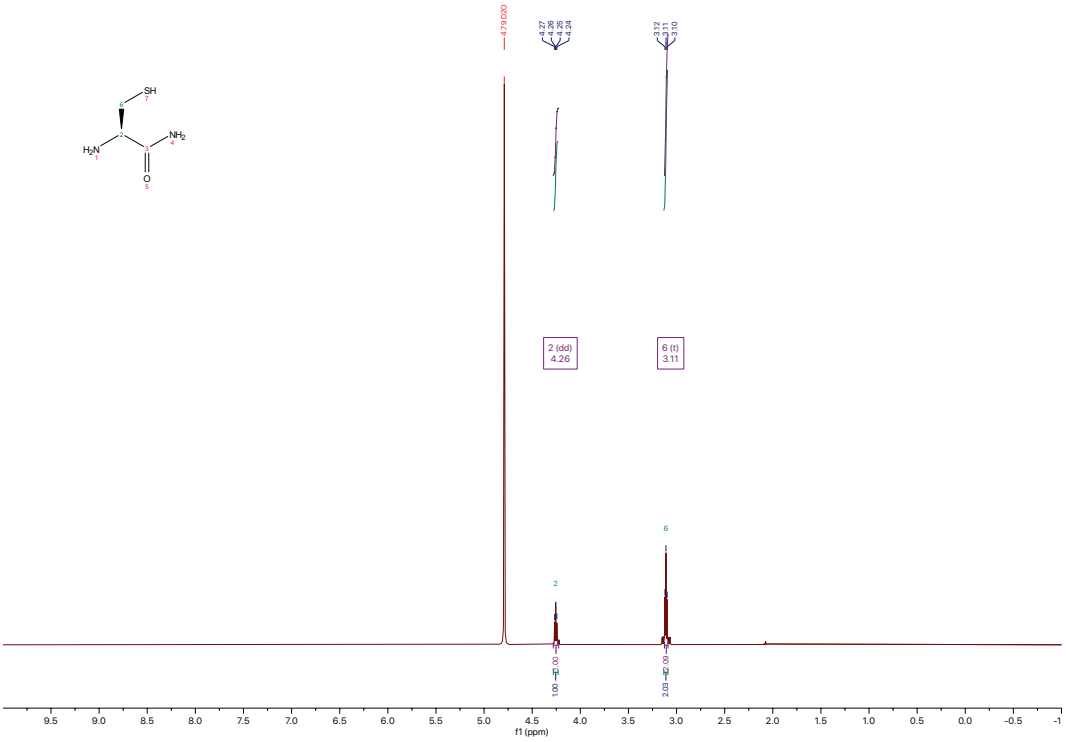


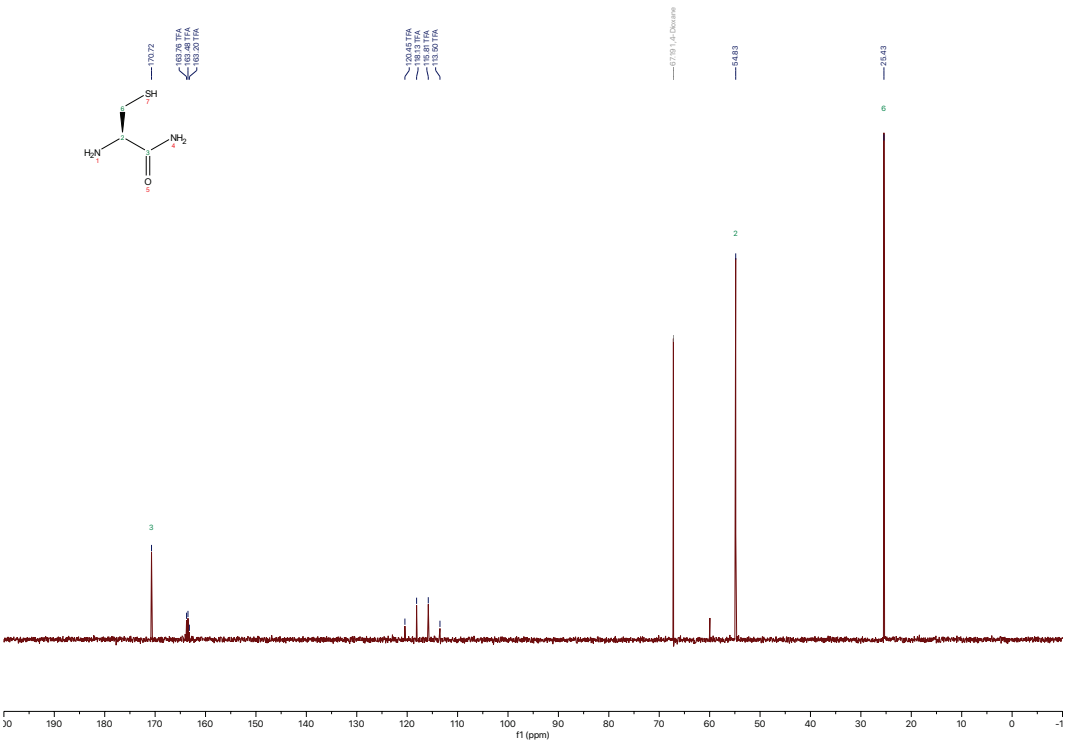
Figure S17. a) RP-HPLC trace (detection at 254 nm) featuring the hydrolysis products of *N*-(benzoyloxy)succinimide after incubation in PBS, pH 7.4, overnight. b) Top: UV trace (detection at 250 nm) obtained upon LC–MS analysis. Middle: Extracted ion chromatogram (*m*/*z* 237.5–238.5) suggests hydrolysis of the *N*-hydroxysuccinimide moiety occurred. Bottom: Extracted ion chromatogram (*m*/*z* 122.5–123.5) suggests benzoic acid as the second hydrolysis product, which results from hydrolysis of the ester.

NMR Spectra

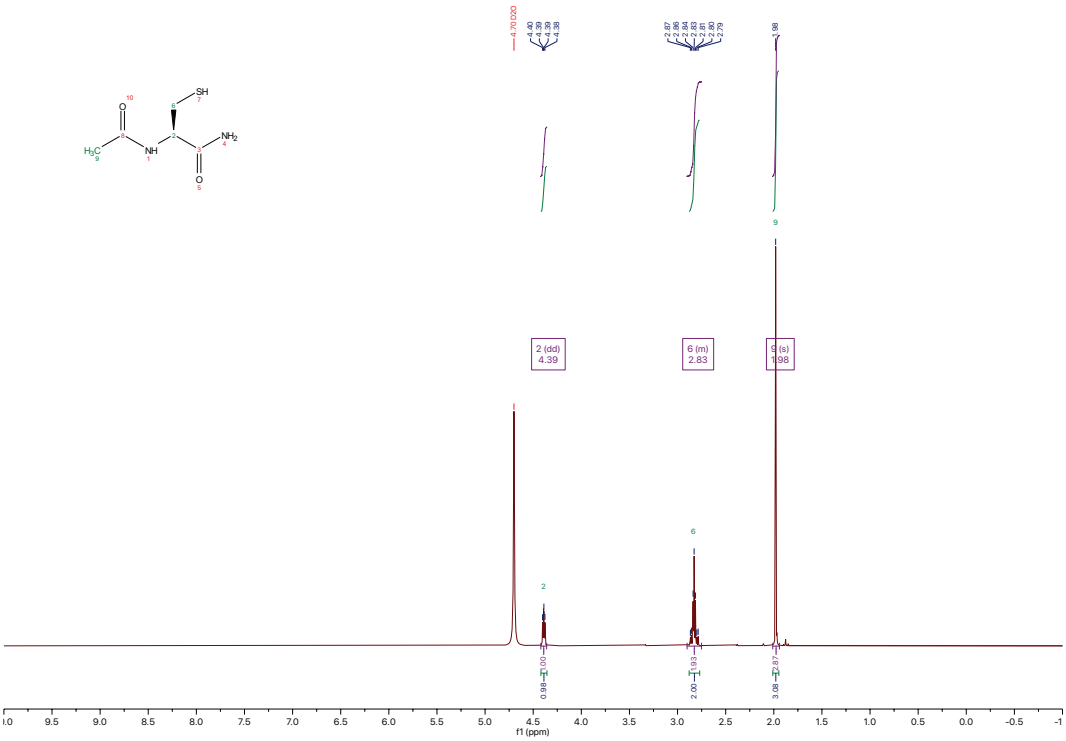
^{1}H NMR Spectrum (500 MHz, D_2O) of H-Cys-NH_2 (TFA salt) (1)



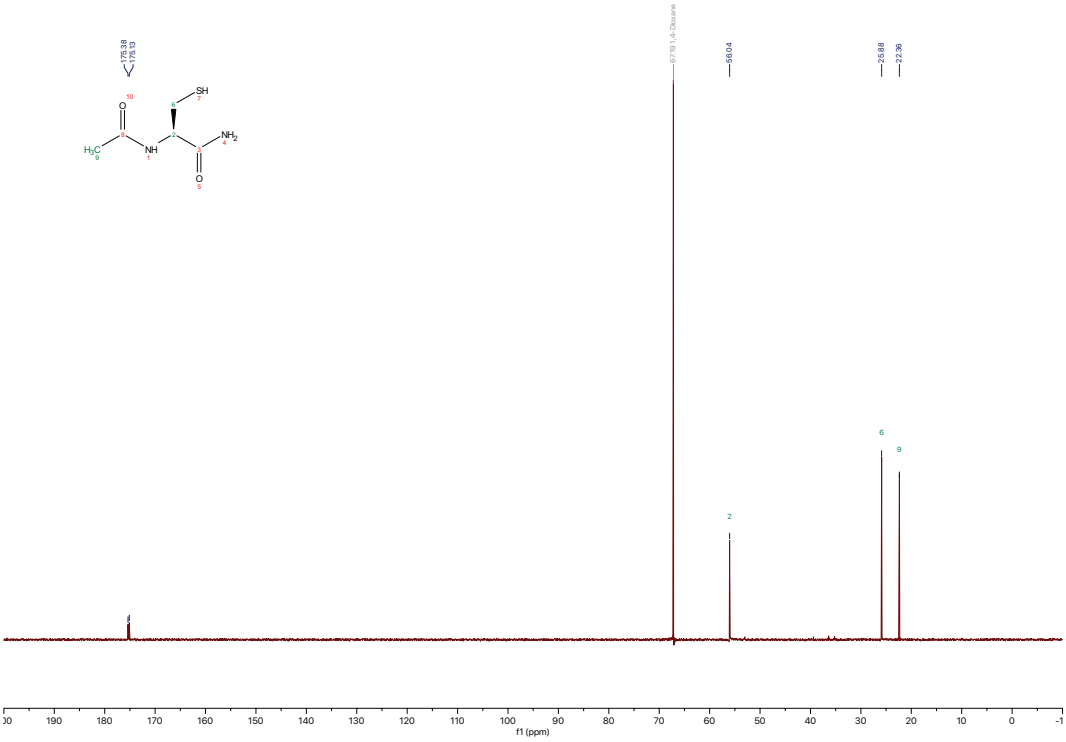
^{13}C NMR Spectrum (126 MHz, D_2O containing 1,4-dioxane for reference) of H-Cys-NH_2 (TFA salt) (1)



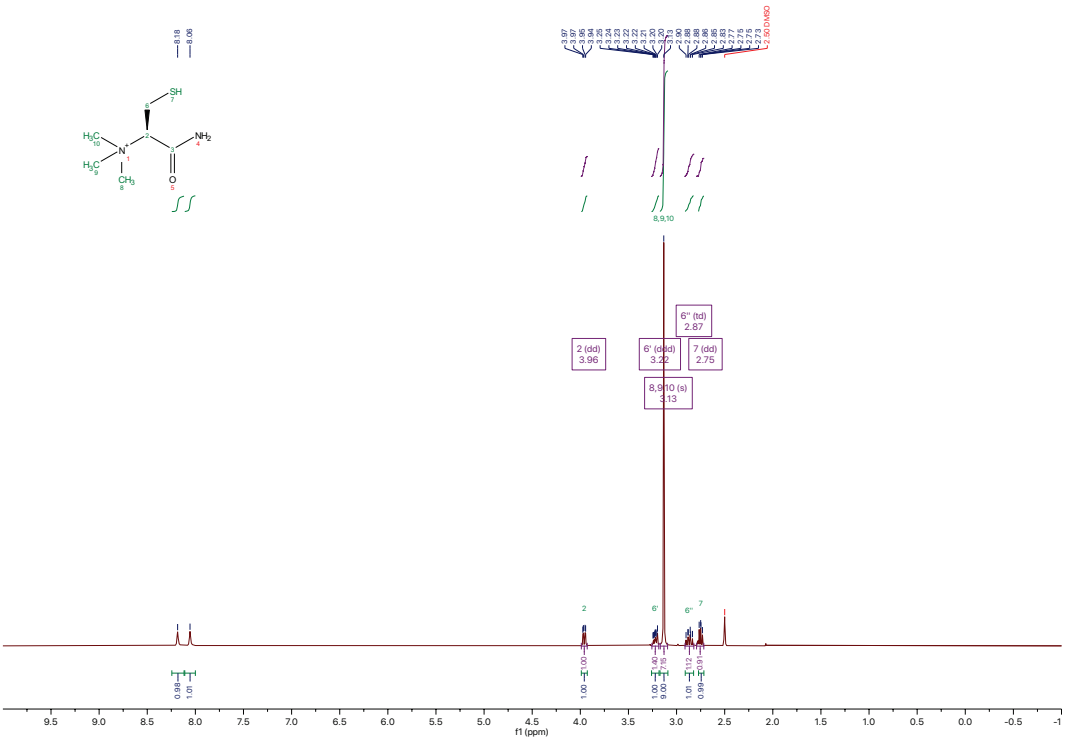
^{1}H NMR Spectrum (500 MHz, D_2O) of Ac-Cys-NH_2 (2)



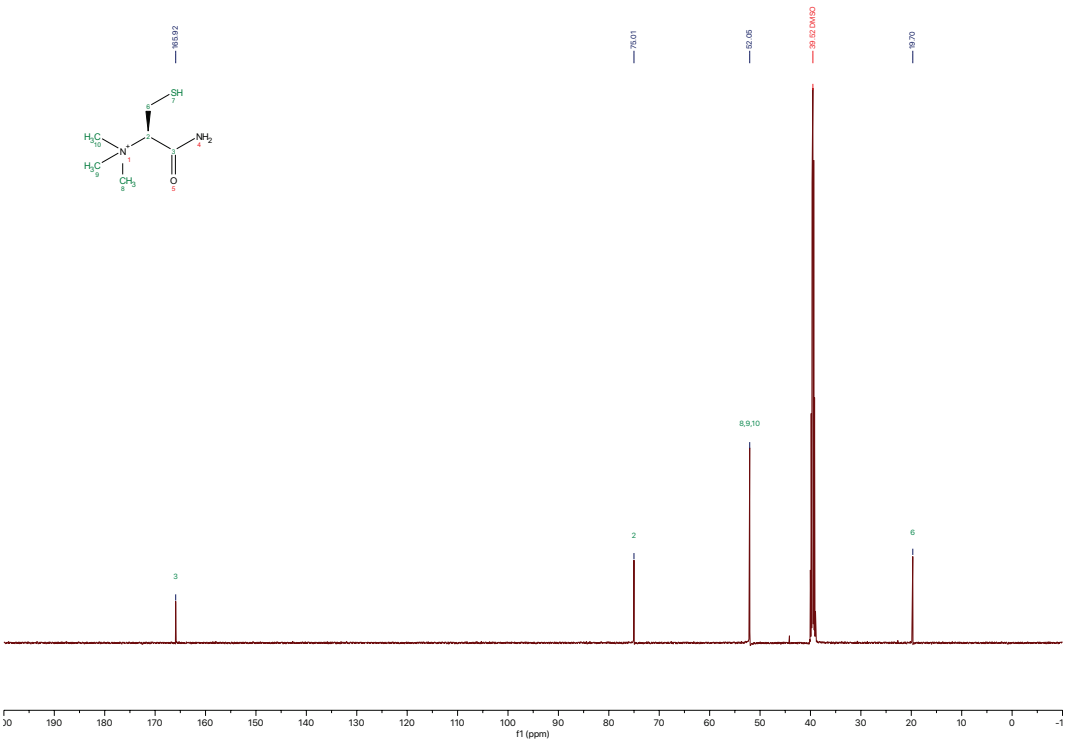
^{13}C NMR Spectrum (126 MHz, D_2O containing 1,4-dioxane for reference) of Ac-Cys-NH_2 (2)



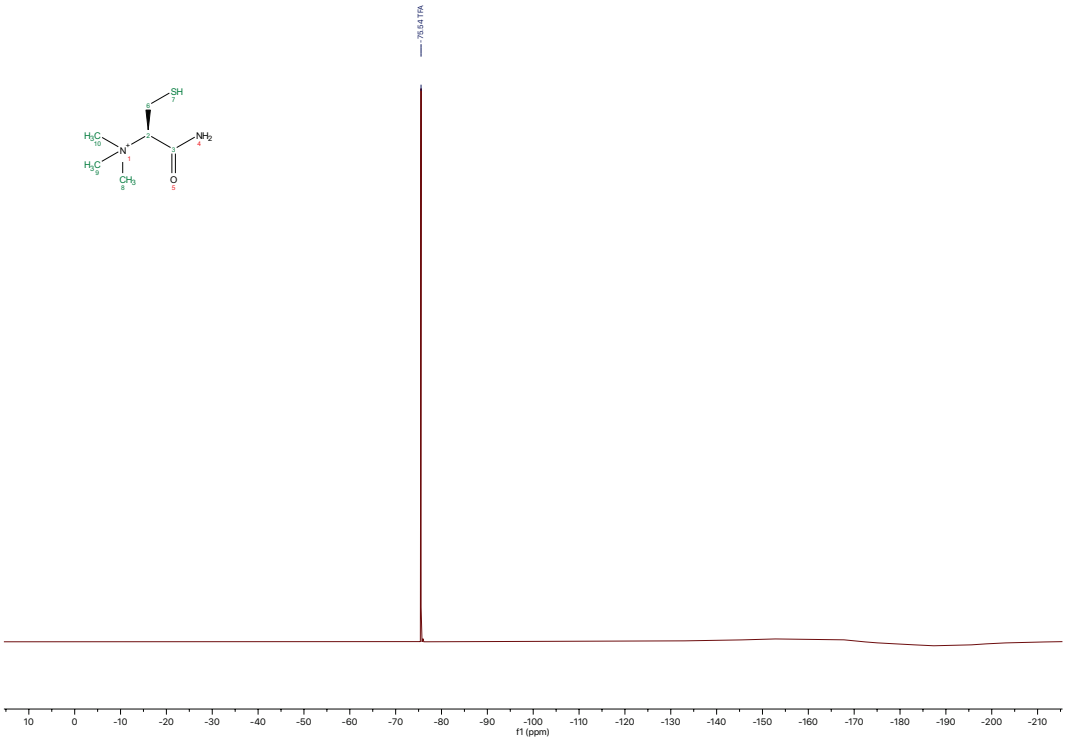
^{1}H NMR Spectrum (500 MHz, DMSO-d_6) of (Me)$_3$-Cys-NH$_2$ (TFA salt) (4)



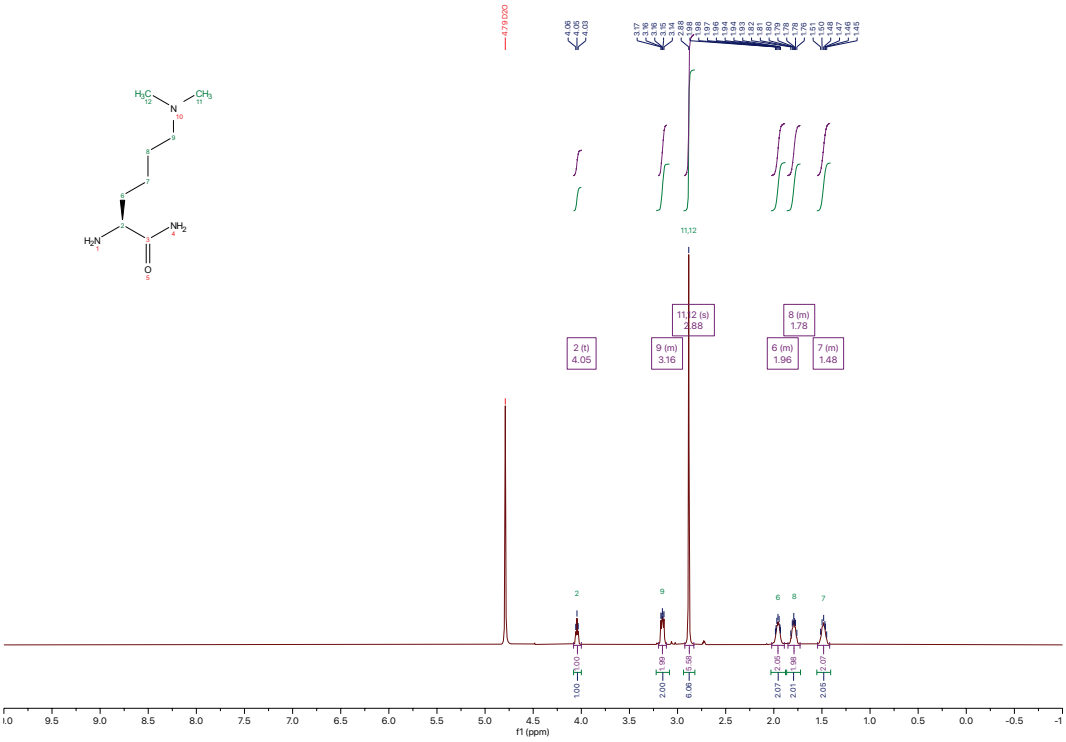
^{13}C NMR Spectrum (126 MHz, DMSO-d_6) of (Me)$_3$-Cys-NH$_2$ (TFA salt) (4)



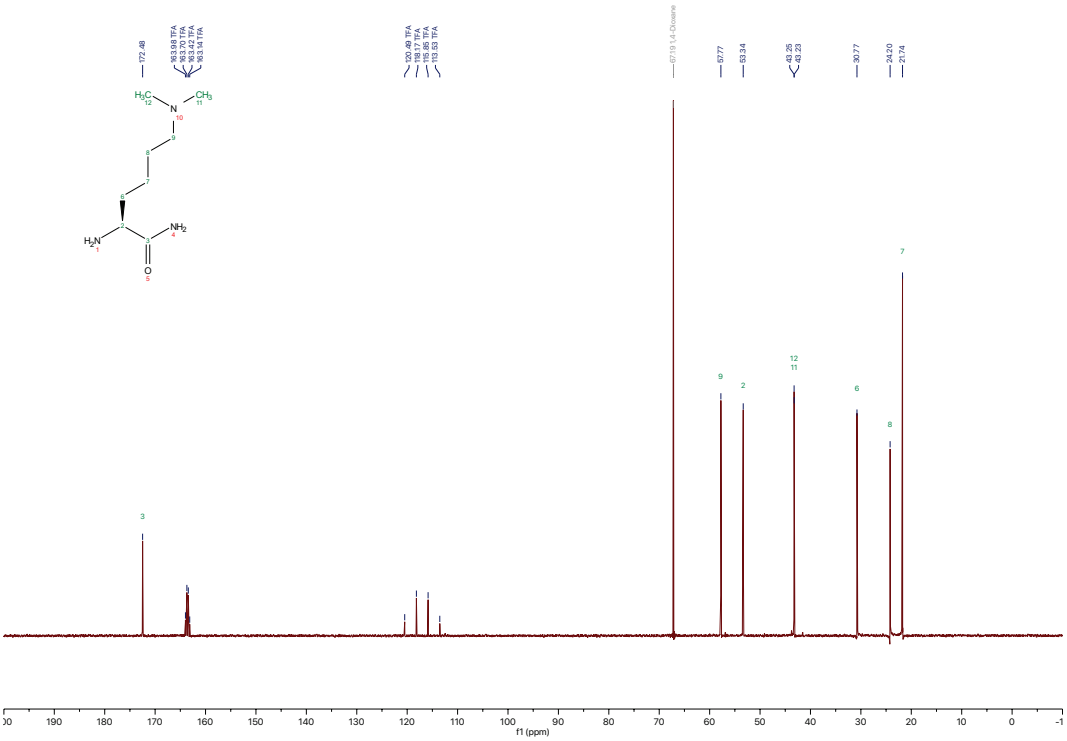
^{19}F NMR Spectrum (471 MHz, D_2O) of $(Me)_3$-Cys-NH_2 (TFA salt) (4)



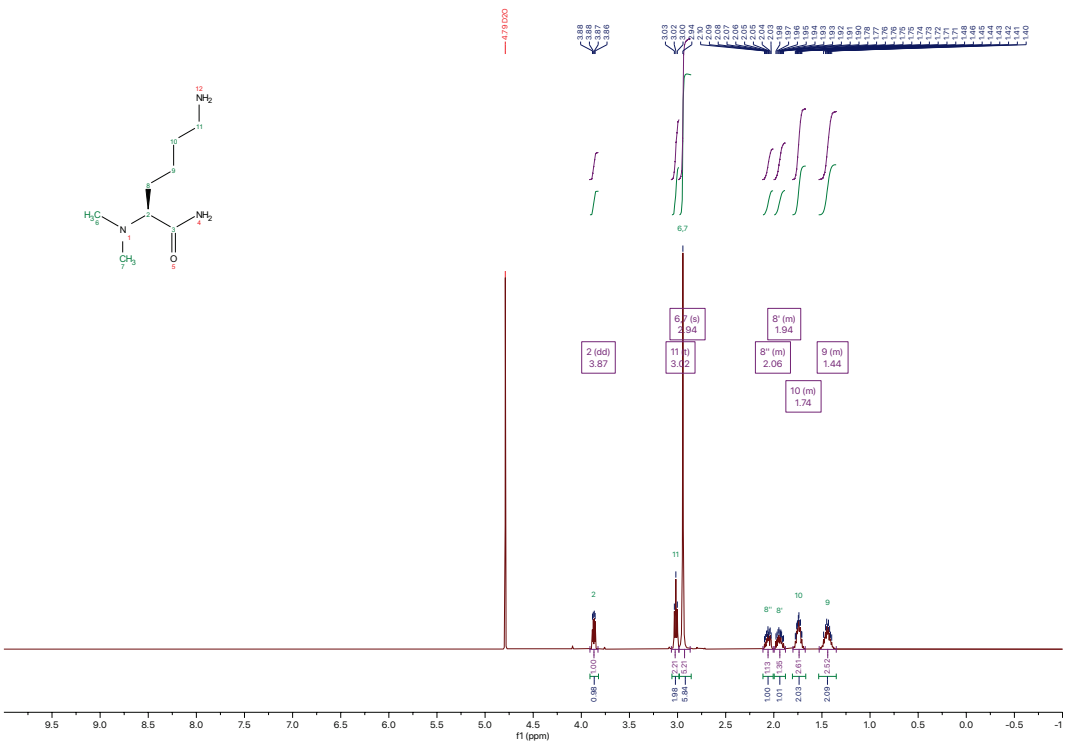
^{1}H NMR Spectrum (500 MHz, D_2O) of H-Lys$(Me)_2$-NH_2 (di-TFA salt) (5)



^{13}C NMR Spectrum (126 MHz, D_2O containing 1,4-dioxane for reference) of H-Lys(Me)$_2$-NH$_2$ (di-TFA salt) (5)

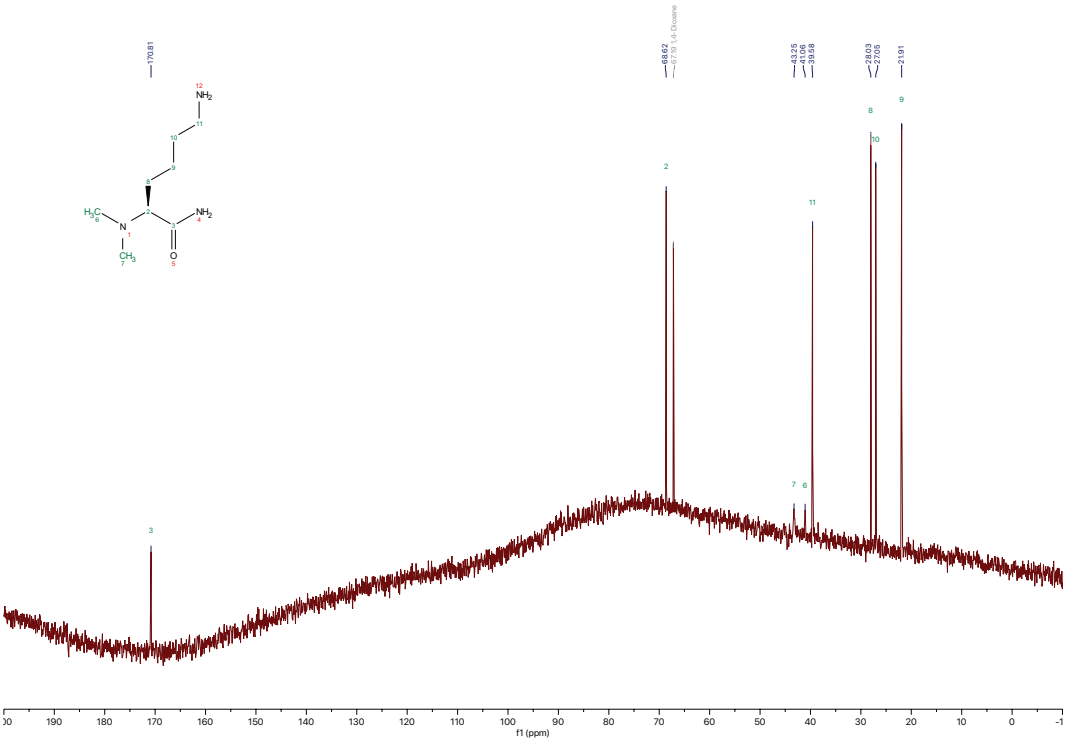


^{1}H NMR Spectrum (500 MHz, D_2O) of (Me)$_2$-Lys-NH$_2$ (di-TFA salt) (6)

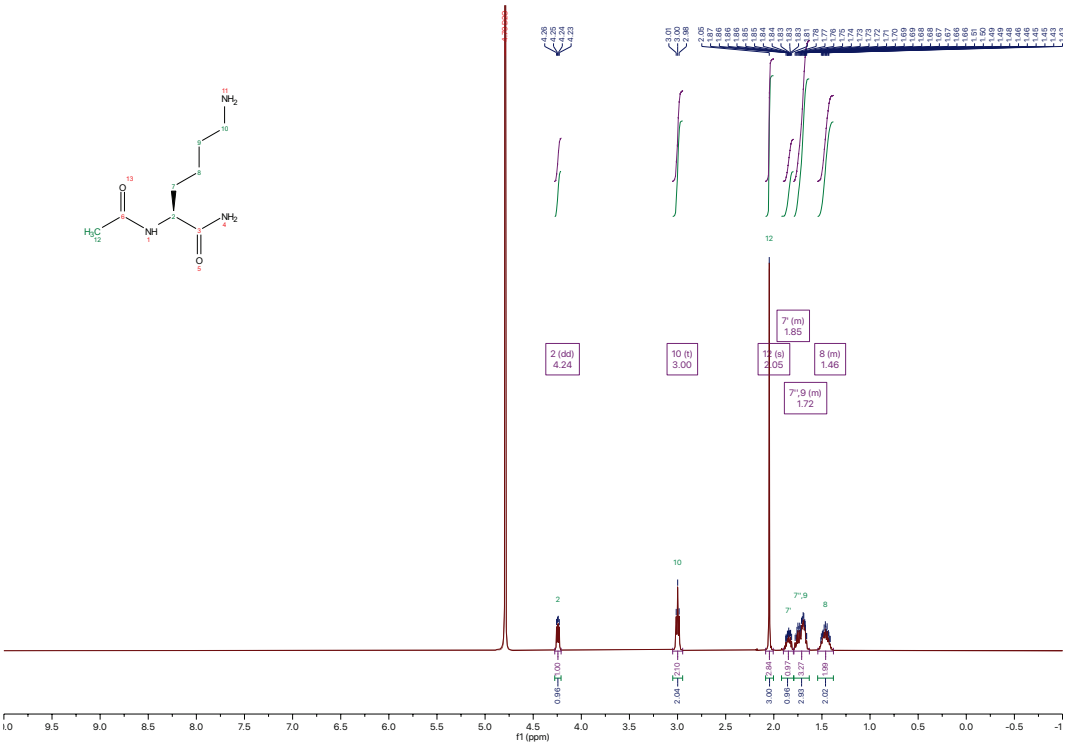


^{13}C NMR Spectrum (126 MHz, D_2O containing 1,4-dioxane for reference) of $(Me)_2$-Lys-NH_2 (di-TFA salt) (6)

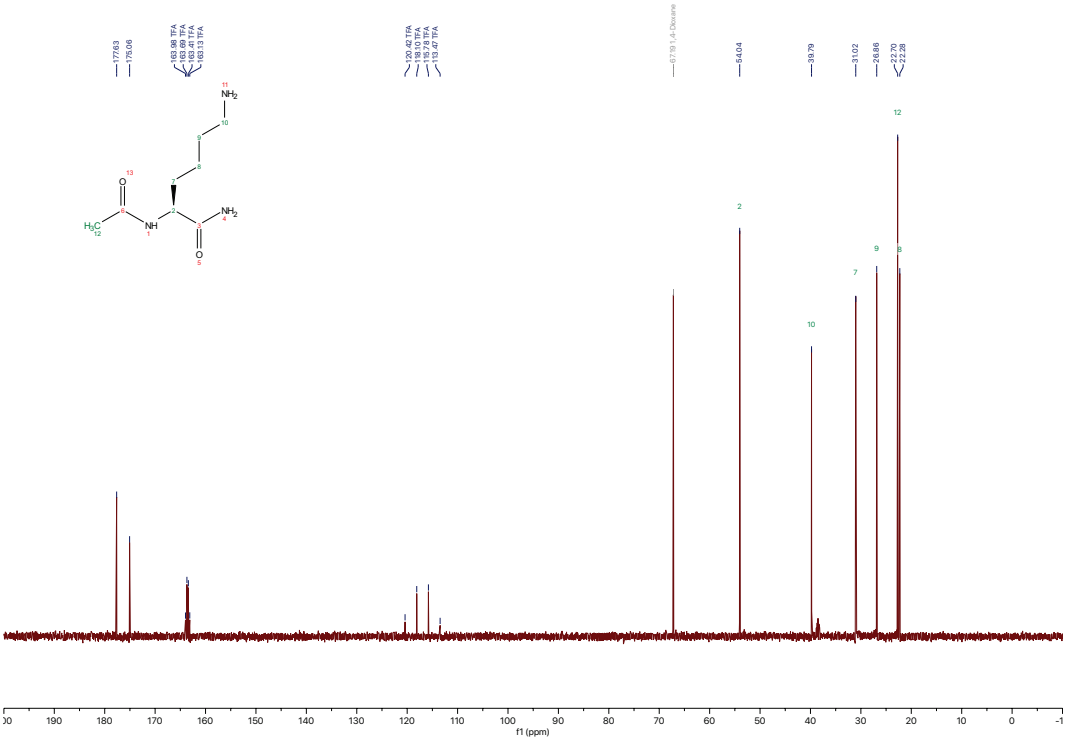
Note: two distinct, broad peaks are observed for the dimethylated α-amine, which suggests restricted rotation around the C–N bond. Distinct, broad peaks were also found for the methyl groups in N^{α},N^{α}-dimethyl-L-lysine.[7]



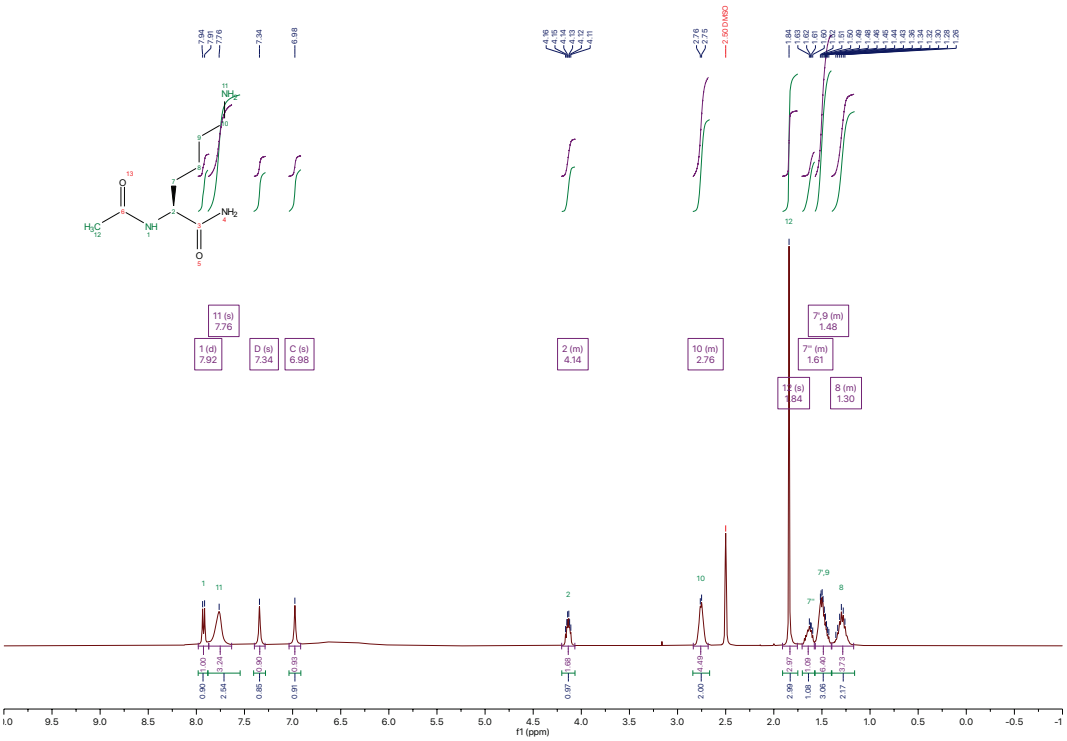
^{1}H NMR Spectrum (500 MHz, D_2O) of Ac-Lys-NH_2 (TFA salt) (7)



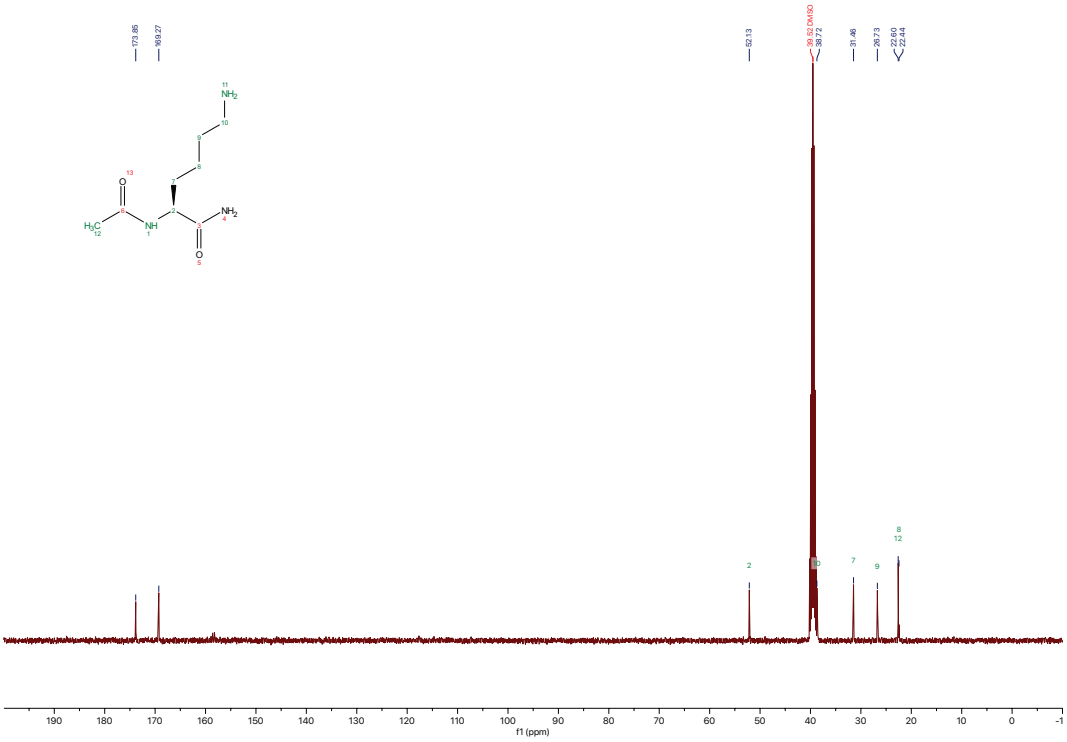
^{13}C NMR Spectrum (126 MHz, D_2O containing 1,4-dioxane for reference) of Ac-Lys-NH_2 (TFA salt) (7)



^{1}H NMR Spectrum (500 MHz, DMSO-d_6) of Ac-Lys-NH$_2$ (TFA salt) (7)



^{13}C NMR Spectrum (126 MHz, DMSO-d_6) of Ac-Lys-NH$_2$ (TFA salt) (7)



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