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Supplementary Materials for

Prebiotic synthesis of cysteine peptides that catalyze peptide ligation in neutral water

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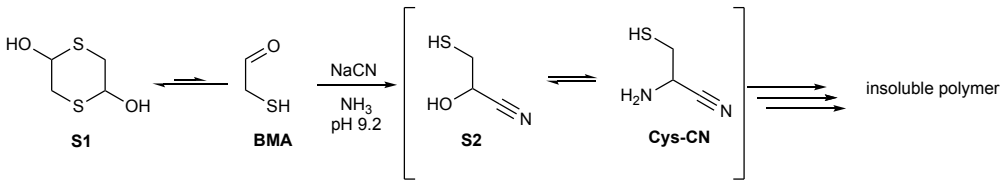
Materials and Methods

Reagents and solvents were obtained and used without further purification, unless specified, from the following commercial sources: *Alfa Aesar, Acros Organics, Apollo Scientific, BDH, Sigma Aldrich, Fluorochem, MerckMillipore, Fisher Scientific, VWR International, Carbosynth, Manchester Organics, Lancaster, Molekula, Honeywell, TCI* and *Santa Cruz Biotechnology*. Sodium hydrosulfide hydrate ($NaSH{\cdot}\times H_2O$ (50%), CAS: 207683-19-0, *Acros Organics*) and sodium sulfide (Na_2S, CAS: 1313-82-2, *Sigma Aldrich*) were used without purification. Deionized water was obtained from an *Elga Option 3* purification system. ^{1}H, ^{13}C and ^{31}P NMR spectra were recorded on *Bruker* NMR spectrometers *AVANCE Neo 700*, *AVANCE III 600*, *AVANCE III 400* and *AVANCE 300*, equipped with a *Bruker* room temperature 5 mm multinuclear gradient probe (700 MHz), 5 mm DCH cryoprobe (600 MHz) and a gradient probe (400 and 300 MHz). All chemical shifts (δ) are reported in parts per million (ppm) relative to residual solvent peaks, and ^{1}H and ^{13}C chemical shifts relative to TMS were calibrated using the residual solvent peak (residual solvent peaks: (δ_H) D_2O – 4.79; DMSO-d_6 – 2.50; $CDCl_3$ – 7.26; CD_3OD – 3.31). Carbon and proton assignments were made using 2D NMR homo- and heteronuclear correlation spectroscopy (^{1}H–^{1}H COSY; ^{1}H–^{13}C HSQC; ^{1}H–^{13}C HMBC). Solvent suppression pulse sequence with presaturation and spoil gradients was used to obtain ^{1}H NMR spectra with solvent suppression (noesygppr1d, *Bruker*) and ^{1}H–^{13}C HMBC NMR spectra (hmbcgplpndprqf, *Bruker*). Coupling constants are reported in Hertz (Hz). Spin multiplicities are indicated by symbols: s (singlet); d (doublet); t (triplet); q (quartet); qn (quintet); spt (septet); oct (octet), m (multiplet); obs. (obscured/coincidental signals), or a combination of these. Diastereotopic geminal (AB) spin systems coupled to one additional nuclei is reported as ABX. NMR data are reported as follows: chemical shift (multiplicity, coupling constants (*J*), number of protons, nuclear assignment). Spectra were recorded at 298 K. Infrared spectra (IR) were recorded on a *Shimadzu IR Tracer 100* FT-IR spectrometer as a solid or neat oil/liquid. Absorption maxima are reported in wavenumber (cm^{-1}). Mass spectra and accurate mass measurements were recorded on a *Waters LCT Premier QTOF* connected to a *Waters Autosampler Manager 2777C*, *Thermo Finnigan MAT900*, and an *Agilent LC* connected to an *Agilent 6510 QTOF* mass spectrometer at the Department of Chemistry, University College London and the UCL School of Pharmacy. Solution pH values were measured using a *Mettler Toledo Seven Compact* pH meter with a *Mettler Toledo InLab* semi-micro pH probe with a *Fisherbrand* FB68801 semi-micro pH probe. The readings for D_2O solutions are reported as pH, and corrected according to *Covington et al. (46)* The readings for H_2O and H_2O/D_2O (9:1) solutions are reported uncorrected.

Attempted Strecker synthesis of cysteine nitrile from β-mercaptoacetaldehyde



A suspension of β-mercaptoacetaldehyde dimer (**S1**; 76 mg, 0.5 mmol), sodium cyanide (74 mg, 1.5 mmol), ammonium hydroxide (384 μL, 5.0 mmol), and monosulfonylmethane (**MSM** (internal standard), 15.6 mg, 0.17 mmol) in water (8 mL) were adjusted to pH 9.2 with conc. HCl. The total volume was adjusted to 10 mL with water and the suspension was stirred vigorously at room temperature. Aliquots (0.5 mL) were removed periodically, centrifuged, and the supernatant analyzed by ^{1}H NMR spectroscopy. The appearance of 2-hydroxy-3-mercaptopropanenitrile (**S2**; 77%) was observed after 20 mins, but was observed to degrade over 24 h (Fig. S1). A low (trace) concentration of **Cys-CN** was observed in the reaction (<5% yield) over 12 h. Analysis after 24 h revealed (upon acute signal amplification (x1000)) a complex slew of resonances in the ^{1}H NMR spectrum. Low abundance of water-soluble material and high level of complexity of ^{1}H NMR resonances were observed – no further attempt was made to characterize this complex mixture. The precipitate was heated at 100 °C in a sealed vial with conc. HCl for 1 h. The precipitate persisted throughout the period of heating in acidic solution. The suspension was cooled, centrifuged, and the supernatant analyzed by NMR spectroscopy. No cysteine derivatives were detected. Our data are consistent with reports that cysteine nitrile **Cys-CN** (as well as cysteine thioesters and cysteine-peptidyl nitriles **Cys-AA-CN**), undergo irrevocable oligomerization and form insoluble polythiazolines (*7*). **Cys-CN** instability has been previously determined following the synthesis of cysteine nitrile trifluoroacetic acid salt (**Cys-CN**·TFA), using conventional synthetic protecting-group chemistry and trifluoroacetic acid deprotection in dichloromethane (*47*). Whilst **Cys-CN** was observed to be stable in highly acidic solutions, incubation of **Cys-CN** at pH 5-6 was observed to rapidly degrade **Cys-CN** and yield insoluble precipitates. These precipitates are likely to be similar to those observed in the Strecker reaction of **S1** that has been described here and elsewhere (*7*, *47, 48*). In contrast, serine nitrile **Ser-CN** (Fig. S2) formed readily (>90%; 2 d) at pH 9.2 and unaltered until monitoring of the reaction mixture ceased (after 6 d).

*Data for 2-hydroxy-3-mercaptopropanenitrile **S2***

^{1}H NMR (600 MHz, H_2O) δ 4.41 (ABX, *J* = 5.6, 9.1 Hz, 1H, (C2)–H), 2.74 (ABX, *J* = 5.6, 12.4 Hz, 1H, (C3)–H), 2.70 (ABX, *J* = 9.1, 12.4 Hz, 1H, (C3)–H′).

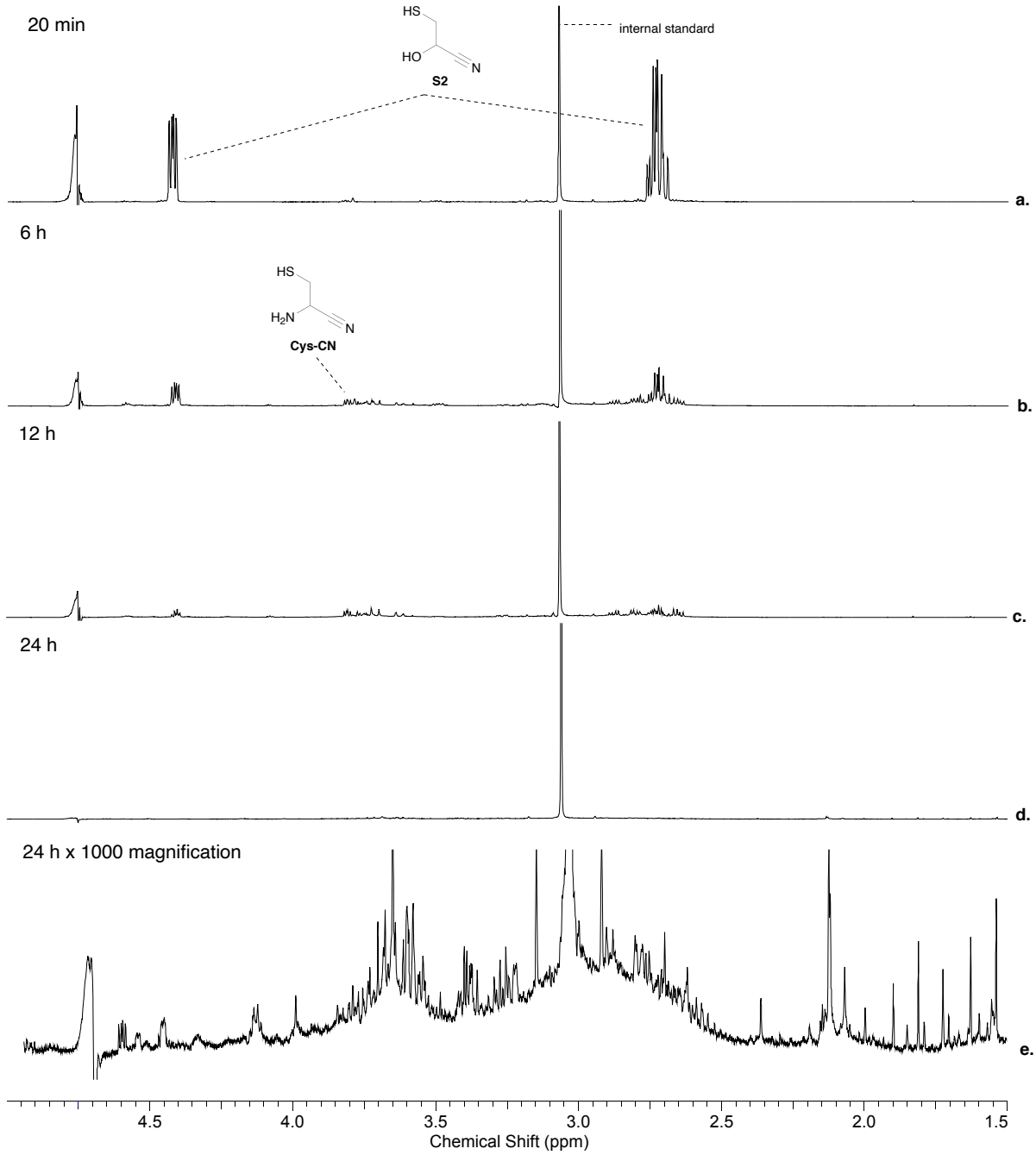
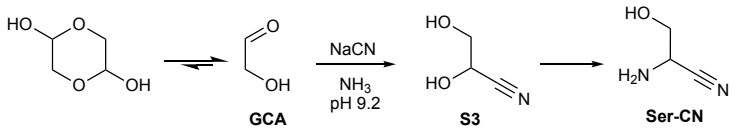


Fig. S1. ^{1}H NMR (600 MHz, H_2O, noesygppr1d, 1.50–5.00 ppm) to show the reaction of β-mercaptoacetaldehyde (**BMA**; 100 mM), sodium cyanide (150 mM) and ammonium hydroxide (500 mM) at pH 9.2 after a. 20 min; b. 6 h; c. 12 h; d. 24 h; e. Spectrum d (24 h), but with increased signal intensity to show the slew of residual baseline resonances. See text for details describing the formation of insoluble polymer due to cysteine nitrile **Cys-CN** self-destruction.

Strecker synthesis of serine nitrile from glycolaldehyde



Glycolaldehyde (**GCA**; 60 mg, 1.0 mmol), sodium cyanide (74 mg, 1.5 mmol), ammonium hydroxide (384 μL, 5.0 mmol), and monosulfonylmethane (**MSM** (internal standard), 15.6 mg, 0.17 mmol) in water (8 mL) were adjusted to pH 9.2 with conc. HCl. The total volume was adjusted to 10 mL with water and the solution was stirred at room temperature. The reaction was monitored by periodic acquisition of NMR spectra until complete conversion of glyceronitrile **S3** to serine nitrile **Ser-CN** (>90%) was observed over 2 d (Fig. S2). The reaction was monitored for a further 4 d to confirm the stability of **Ser-CN** at pH 9.2. In contrast, the attempted Strecker synthesis of cysteine nitrile **Cys-CN** (Fig. S1) from β-mercaptoacetaldehyde resulted in the formation of insoluble precipitates.

*Data for glyceronitrile **S3***

^{1}H NMR (600 MHz, H_2O) δ 4.64 (t, *J* = 5.0 Hz, 1H, (C2)–H), 3.75 (d, *J* = 5.0 Hz, 2H, (C3)–H_2).

*Data for serine nitrile **Ser-CN***

^{1}H NMR (600 MHz, H_2O) δ 3.92 (ABX, *J* = 4.7, 5.8 Hz, 1H, (C2)–H), 3.76 (ABX, *J* = 4.7, 11.4 Hz, 1H, (C3)–H), 3.68 (ABX, *J* = 5.8, 11.4 Hz, 1H, (C3)–H′).

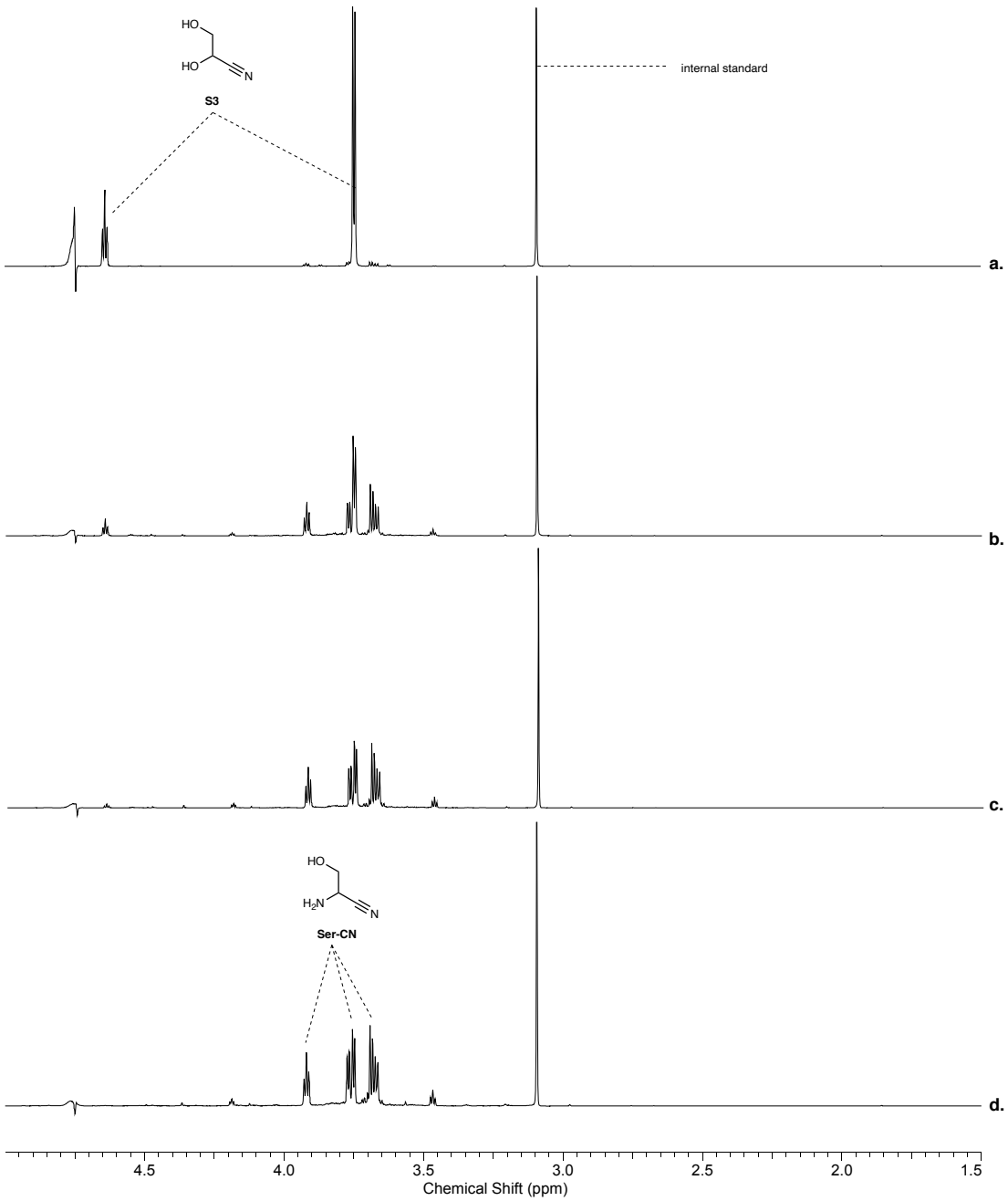
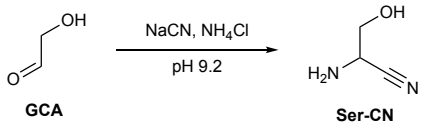


Fig. S2. ^{1}H NMR (600 MHz, H_2O, noesygppr1d, 1.50–5.00 ppm) to show the reaction of glycolaldehyde (100 mM), sodium cyanide (150 mM) and ammonium hydroxide (500 mM) at pH 9.2 after a. 30 min; b. 24 h; c. 48 h; d. 6 days.

Synthesis of α-amidocysteines from α-amidodehydroalanine nitriles

Preparative synthesis and isolation of DL-serine nitrile



GCA **Ser-CN**

Glycolaldehyde **GCA** (1.20 g, 20.0 mmol), sodium cyanide (1.08 g, 22.0 mmol) were dissolved in H_2O/D_2O (9:1; 15 mL). Ammonium chloride (5.35 g, 100 mmol) was added and the solution was adjusted to pH 9.2 with HCl/NaOH, followed by adjustment of the total reaction volume to 20 mL. The reaction was monitored by periodic acquisition of NMR spectra until complete conversion of glyceronitrile to serine nitrile **Ser-CN** (>95%) was observed. The reaction was concentrated *in vacuo* and the residue repeatedly triturated with methanol until **Ser-CN** was completely recovered from the solid. The organics were then concentrated *in vacuo* to give serine nitrile **Ser-CN** as a yellow oil (680 mg), which was used without further purification. ^{1}H NMR (700 MHz, D_2O, noesygppr1d) δ 3.97 (dd, *J* = 4.7, 5.8 Hz, 1H, (C2)–H), 3.82 (ABX, *J* = 4.7, 11.4 Hz, 1H, (C3)–H), 3.74 (ABX, *J* = 5.8, 11.4 Hz, 1H, (C3)–H′). ^{13}C NMR (176 MHz, D_2O) δ 121.9 (C1), 63.3 (C3), 45.3 (C2). HRMS-ESI $[M+H^+]^+$ calculated for formula $C_3H_7N_2O^+$, 87.0553; found 87.0558. IR (cm^{-1}): 3331, 3280, 3182, 2935, 2838, 2233, 1672, 1606.

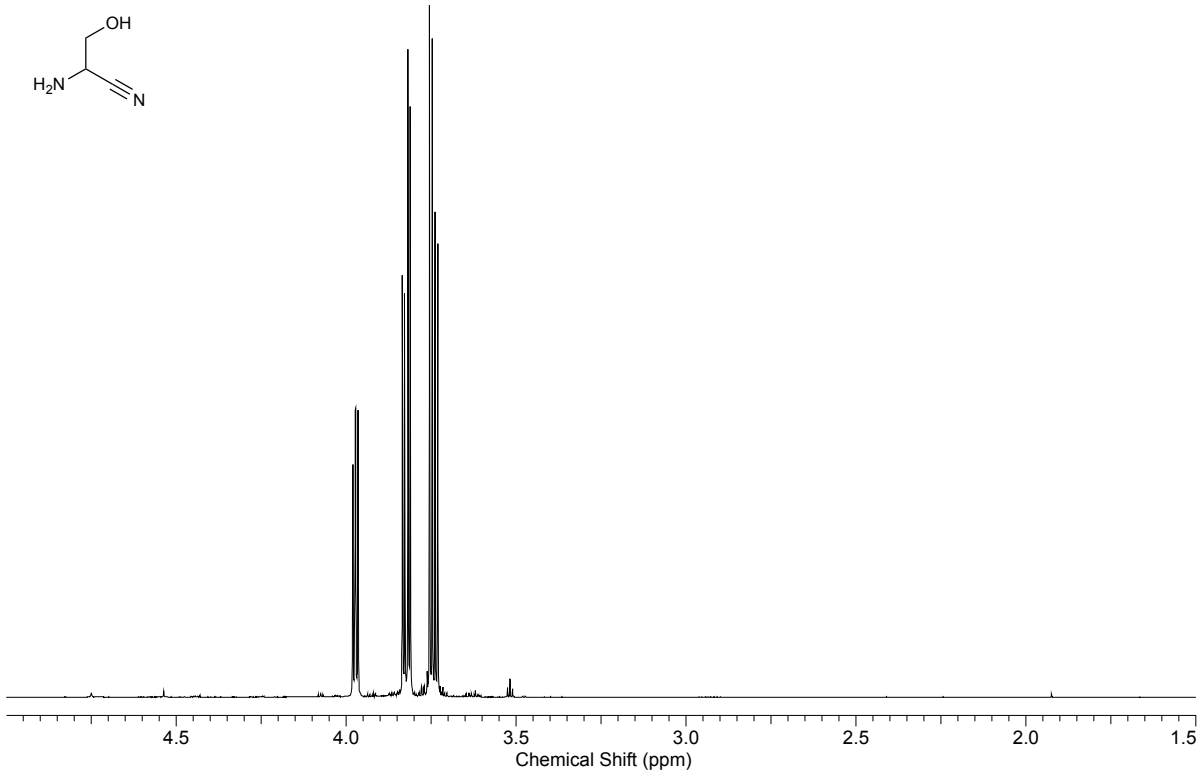


Fig. S3. ^{1}H NMR (700 MHz, D_2O, noesygppr1d, 1.50 – 5.00 ppm) spectrum to show **Ser-CN**.

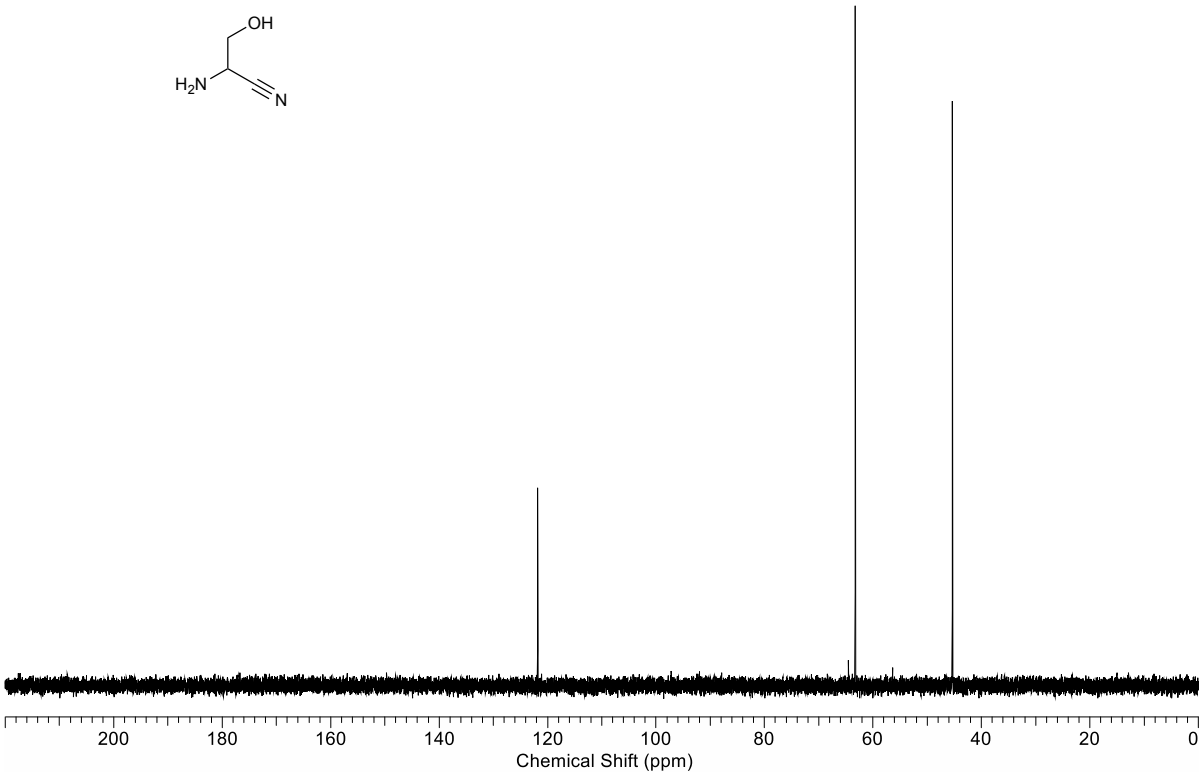
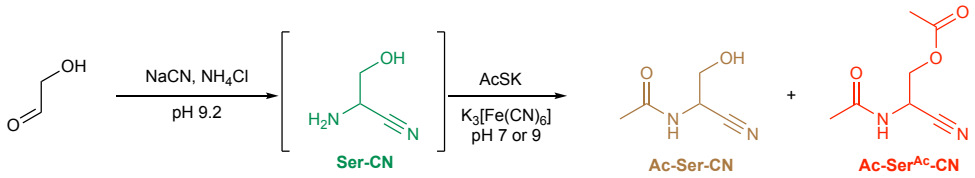


Fig. S4. ^{13}C NMR (176 MHz, D_2O 0 – 220 ppm) spectrum to show **Ser-CN**.

Acetylation of DL-serine nitrile with thioacetate

Acetylation of crude DL-serine nitrile with thioacetate



Glycolaldehyde **GCA** (1 M), sodium cyanide (1.2 equiv) and ammonium chloride (5 equiv.) were dissolved in water and the solution was adjusted to pH 9.2 by addition of HCl/NaOH. The solution was then stirred at room temperature. After 24 h the reaction was diluted twenty-fold with water ([**Ser-CN**] = 50 mM). Potassium thioacetate (3 equiv.; 150 mM) was added and the solution adjusted to pH 7.0 or pH 9.0 by addition of 4 M HCl/NaOH. Potassium hexacyanoferrate(III) ($K_3[Fe(CN)_6]$; 9 equiv.; 450 mM) was added, and the solution was stirred at room temperature whilst maintaining the solution at pH 7.0 or pH 9.0 with 4 M HCl/NaOH. After 20 min the reaction was centrifuged, and the supernatant analysed by NMR spectroscopy. Yields are reported in Table S1, and NMR spectra are shown in Fig. S5.

pH	Amount (%)		
	Ac-Ser-CN	Ac-Ser^{Ac}-CN	Ser-CN
7	70	10	7
9	>95	n.d	n.d

Table S1. Yields of acetylation of crude **Ser-CN** (50 mM; produced from the reaction of glycolaldehyde (**GCA**), sodium cyanide (NaCN) and ammonia (NH_3) at pH 9.2) after reaction with **AcSK** (150 mM) and potassium hexacyanoferrate(III) ($K_3[Fe(CN)_6]$ (450 mM) at pH 7 or pH 9 and room temperature. n.d = not detected by ^{1}H NMR spectroscopy.

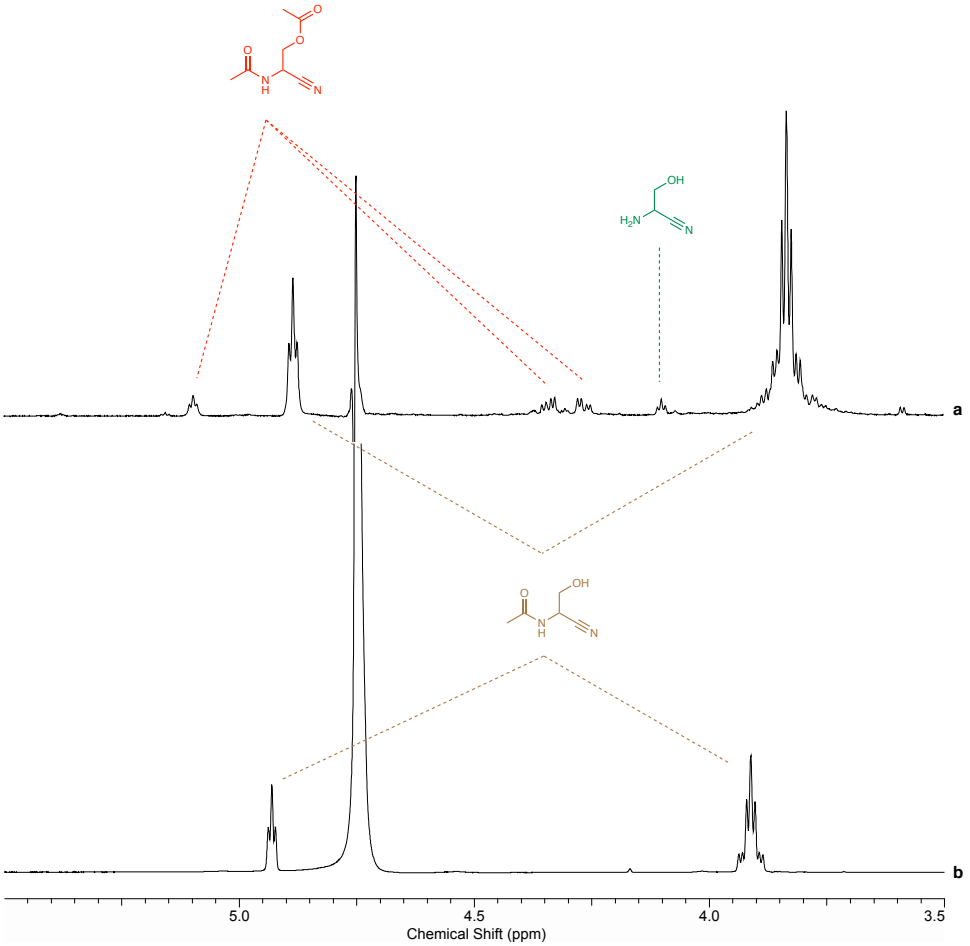
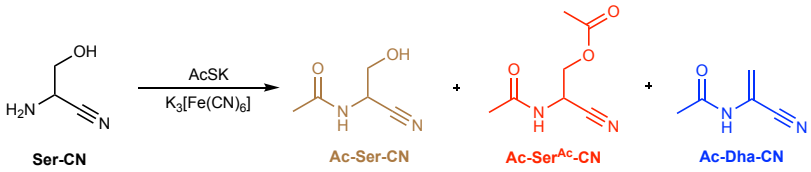


Fig. S5. ^{1}H NMR spectra to show the acetylation of crude **Ser-CN** with potassium thioacetate (**AcSK**) and potassium hexacyanoferrate(III) ($K_3[Fe(CN)_6]$). Glycolaldehyde (**GCA**; 1 M), NaCN (1.2 equiv.) NH_4Cl (5 equiv.) were dissolved in water at pH 9.2 and stirred at room temperature for 24 h. The reaction was diluted twenty-fold with water to give crude **Ser-CN** (50 mM), which was reacted with **AcSK** (3 equiv.) and ($K_3[Fe(CN)_6]$; 9 equiv.) at **a.** pH 7.0 after 20 min (600 MHz, H_2O, noesygppr1d, 3.50–5.50 ppm), and **b.** pH 9.0 after 20 min (700 MHz, H_2O, noesygppr1d, 3.50–5.50 ppm).

Acetylation of DL-serine nitrile with thioacetate



DL-Serine nitrile **Ser-CN** (50 – 100 mM) and potassium thioacetate (**AcSK**, 3 – 10 equiv.)) in water were adjusted to pH 7.0 or pH 9.0 by addition of 4 M HCl/NaOH. Potassium hexacyanoferrate(III) (9 – 20 equiv.) was added, and the solution was stirred at room temperature whilst maintaining the pH at 7.0 or 9.0 with 4 M HCl/NaOH. After 1 h the reaction was centrifuged, and the supernatant analysed by NMR spectroscopy. Yields are reported in Table S2, and NMR spectra are shown in Fig. S6 ([**Ser-CN**] = 50 mM) and Fig. S7 ([**Ser-CN**] = 100 mM).

Ser-CN/mM	**pH**	**AcSK (equiv.)**	**$K_3[Fe(CN)_6]$ (equiv.)**	**Amount (%)**		
				Ac-Ser-CN	**Ac-Ser^{Ac}-CN**	**Ac-Dha-CN**
50	7	3	9	43	53	n.d
50	9	3	9	42	52	n.d
100	7	5	10	18	71	6
100	7	10	20	<5%	91	n.d

Table S2. Yields of acetylation of **Ser-CN** after reaction with **AcSK** and potassium hexacyanoferrate(III) ($K_3[Fe(CN)_6]$ at pH 7 and room temperature. n.d = not detected by ^{1}H NMR spectroscopy.

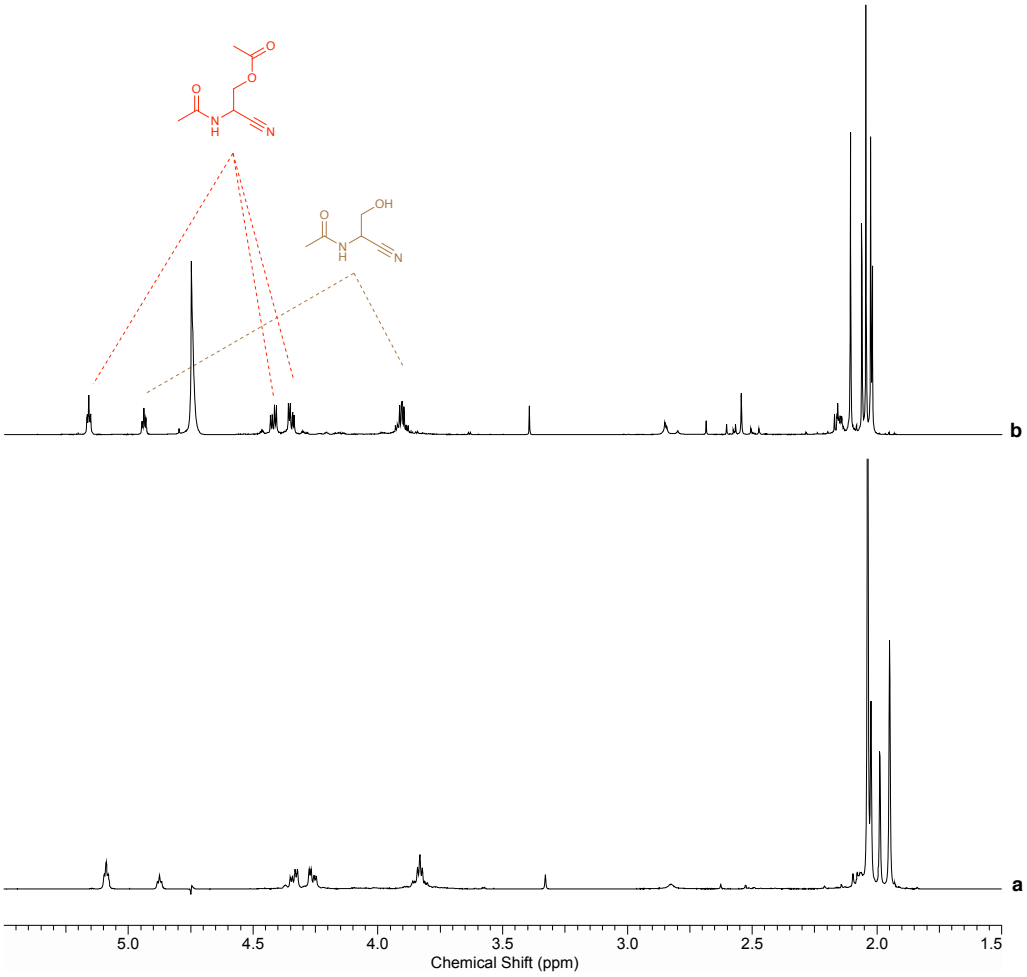


Fig. S6. ^{1}H NMR spectra to show the reaction of **Ser-CN** (50 mM) with potassium thioacetate (3 equiv.) and potassium hexacyanoferrate(III) ($K_3[Fe(CN)_6]$; 9 equiv.) at **a.** pH 7.0 (600 MHz, H_2O, noesygppr1d, 1.50–5.50 ppm, and **b.** pH 9.0 (700 MHz, H_2O, noesygppr1d, 1.50–5.50 ppm).

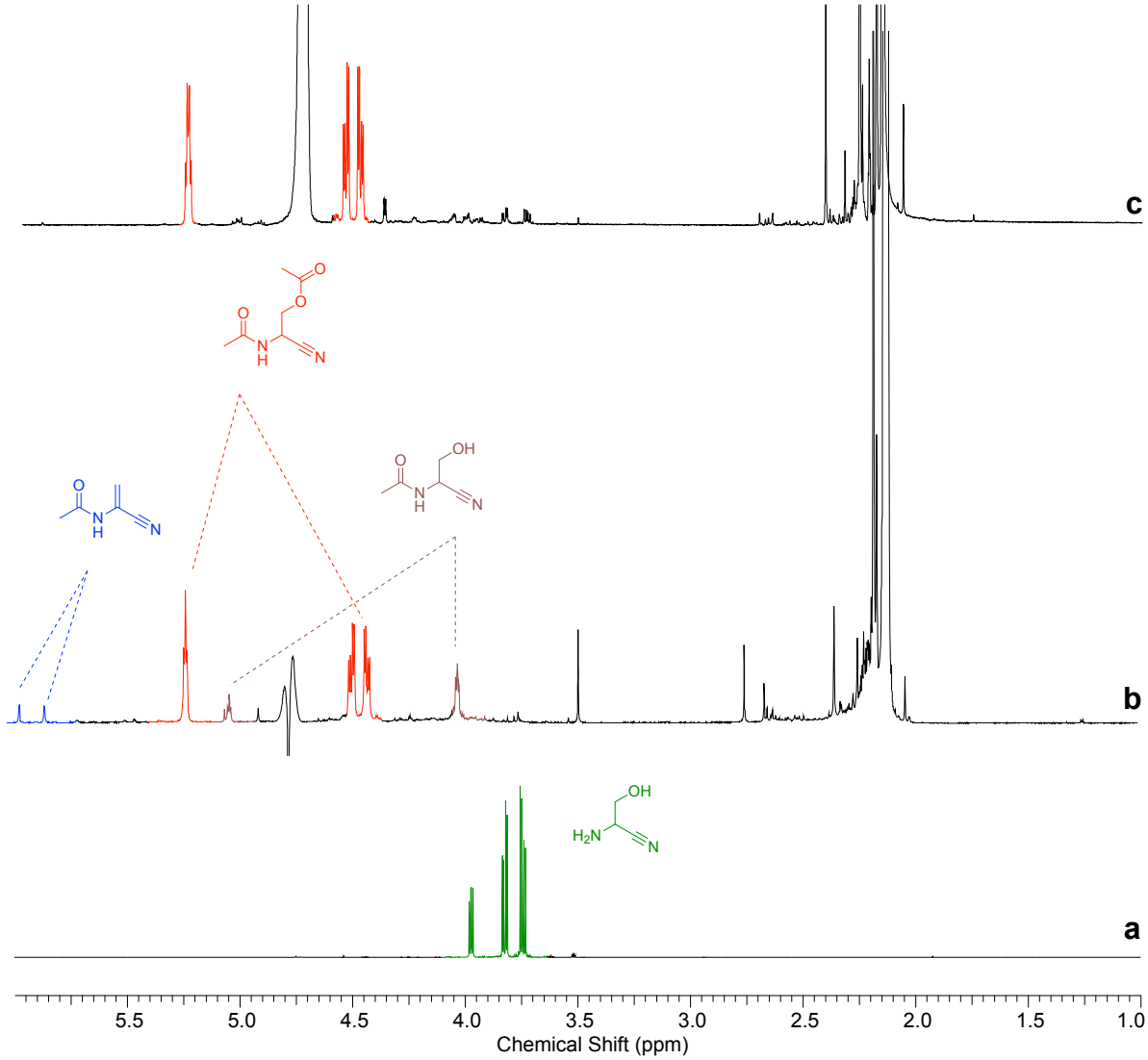
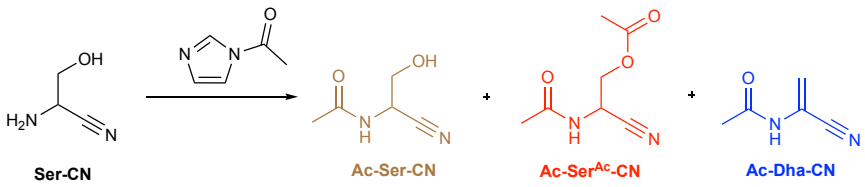


Fig. S7. ^{1}H NMR spectra (700 MHz, H_2O, noesygppr1d, 1.00–6.00 ppm) to show: a) DL-serine nitrile **Ser-CN** at pH 9.2 and room temperature; b) the reaction of **Ser-CN** (100 mM) with potassium thioacetate (5 equiv.) and potassium hexacyanoferrate(III) ($K_3[Fe(CN)_6]$;10 equiv.) at pH 7.0. c) the reaction of **Ser-CN** (100 mM) with potassium thioacetate (10 equiv.) and potassium hexacyanoferrate(III) ($K_3[Fe(CN)_6]$; 20 equiv.) at pH 7.0. The appearance of **Ac-Dha-CN** in spectrum b. was attributed to temporary sampling of the reaction at pH 8.0 during stabilisation of the reaction pH at 7 prior to NMR analysis.

Acetylation of DL-serine nitrile with N-acetylimidazole



A solution of DL-serine nitrile **Ser-CN** (100 mM) was adjusted to pD 7 with 4 M DCl/NaOD. *N*-Acetylimidazole **NAI** (2.5–10 equiv.) was added and the solution readjusted to pD 7. The reaction was monitored by NMR spectroscopy at room temperature. Product distributions are reported in Table S3. See Fig. S8 and Fig. S9 for representative NMR spectra, and a ^{1}H–^{13}C HMBC NMR spectrum (Fig. S10) to confirm the assignment of the intermediate *O*-acetyl-DL-serine nitrile **Ser^{Ac}-CN**.

*Data for O-acetyl-DL-serine nitrile **Ser^{Ac}-CN***

1H NMR (700 MHz, D_2O) δ 4.29 (ABX, *J* = 5.1, 11.2 Hz, 1H, (C3)–H), 4.22 (ABX, *J* = 5.1, 11.2 Hz, 1H, (C3)–H′), 4.16 (dd, *J* = 5.1, 5.1 Hz, 1H, (C2)–H), 2.12 (obs. s, 3H, $COCH_3$).

*Data for N,O-diacetyl-DL-serine nitrile **Ac-Ser^{Ac}-CN***

1H NMR (700 MHz, D_2O) δ 5.09 (dd, *J* = 4.9, 5.0 Hz, 1H, (C2)–H), 4.37 (ABX, *J* = 5.4, 11.7 Hz, 1H, (C3)–H), 4.31 (dd, J = 4.9, 11.7 Hz, 1H, (C3)–H′), 2.10 (s, 3H, $COCH_3$), 1.98 (s, 3H, $COCH_3$).

*Data for N-acetyl-DL-serine nitrile **Ac-Ser-CN***

1H NMR (700 MHz, D_2O) δ 4.82 (dd, *J* = 5.4, 5.4 Hz, 1H, (C2)–H), 3.82 (ABX, *J* = 5.4, 11.4 Hz, 1H, (C3)–H), 3.79 (ABX, *J* = 5.4, 11.4 Hz, 1H, (C3)–H′), 1.99 (s, 3H, $COCH_3$).

*Data for N-acetyldehydroalanine nitrile **Ac-Dha-CN** (partial assignment)*

1H NMR (700 MHz, D_2O) δ 5.80 (d, *J* = 1.6 Hz, 1H, (C3)–H), 5.69 (d, *J* = 1.6 Hz, 1H, (C3)–H′).

NAI (equiv.)	Time (h)	Amount (%)		
		Ac-Ser-CN	Ac-Ser^{Ac}-CN	Ac-Dha-CN
2.5	2	16	40	n.d
2.5	19	24	37	5
2.5	64	25	28	11
5	2	6	78	n.d
5	19	5	72	6
5	64	6	68	10
10	4	4	91	2

Table S3. Yields of acetylation of **Ser-CN** (100 mM) after reaction with *N*-acetylimidazole **NAI** (2.5 – 10 equiv.) at pD 7 and room temperature. n.d = not detectable by ^{1}H NMR spectroscopy.

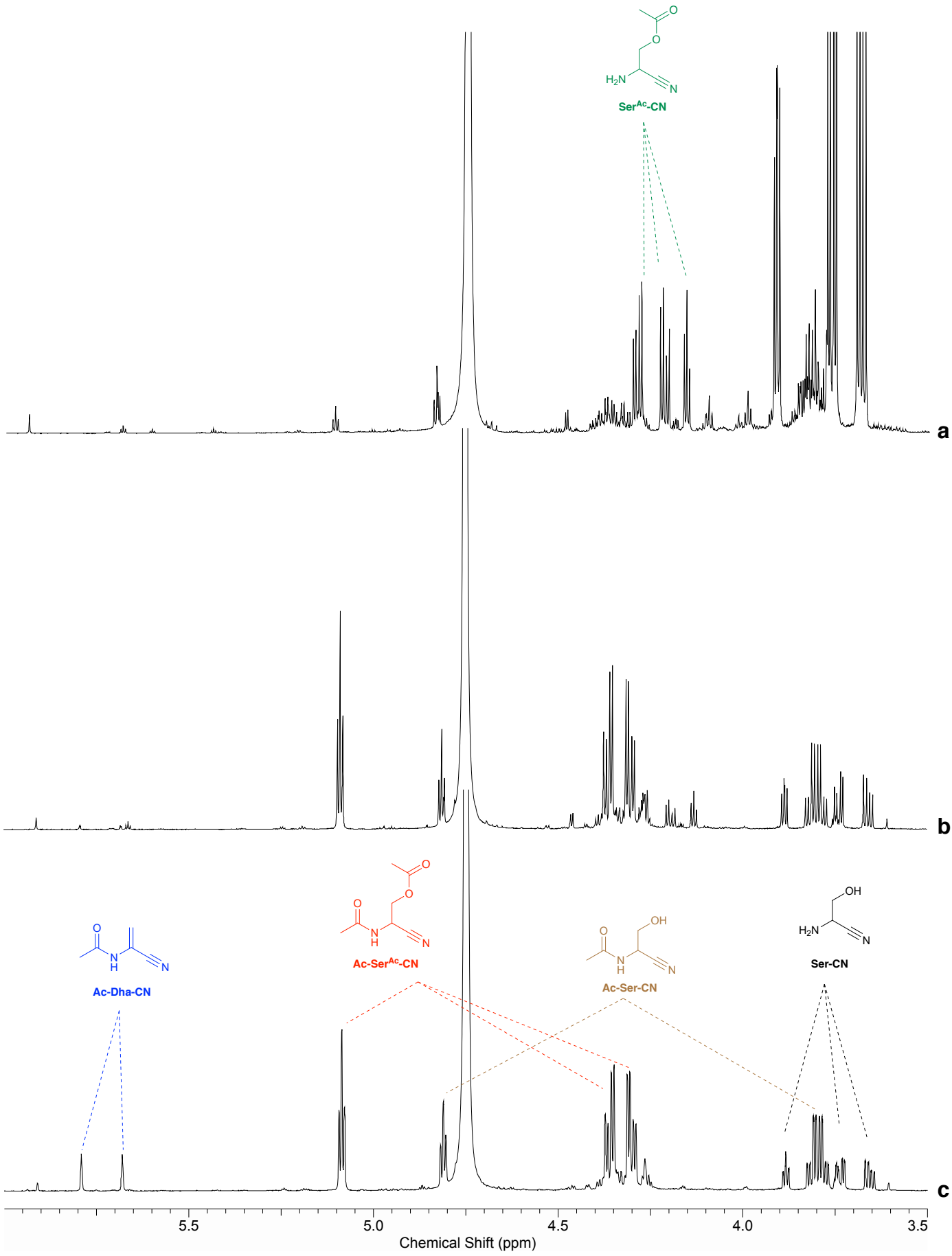


Fig. S8. ^{1}H NMR spectra (700 MHz, D_2O, 3.50–6.00 ppm) to show the reaction of **Ser-CN** (100 mM) with *N*-acetylimidazole **NAI** (250 mM) at pD 7 and room temperature after a) 5 min; b) 2 h; c) 19 h.

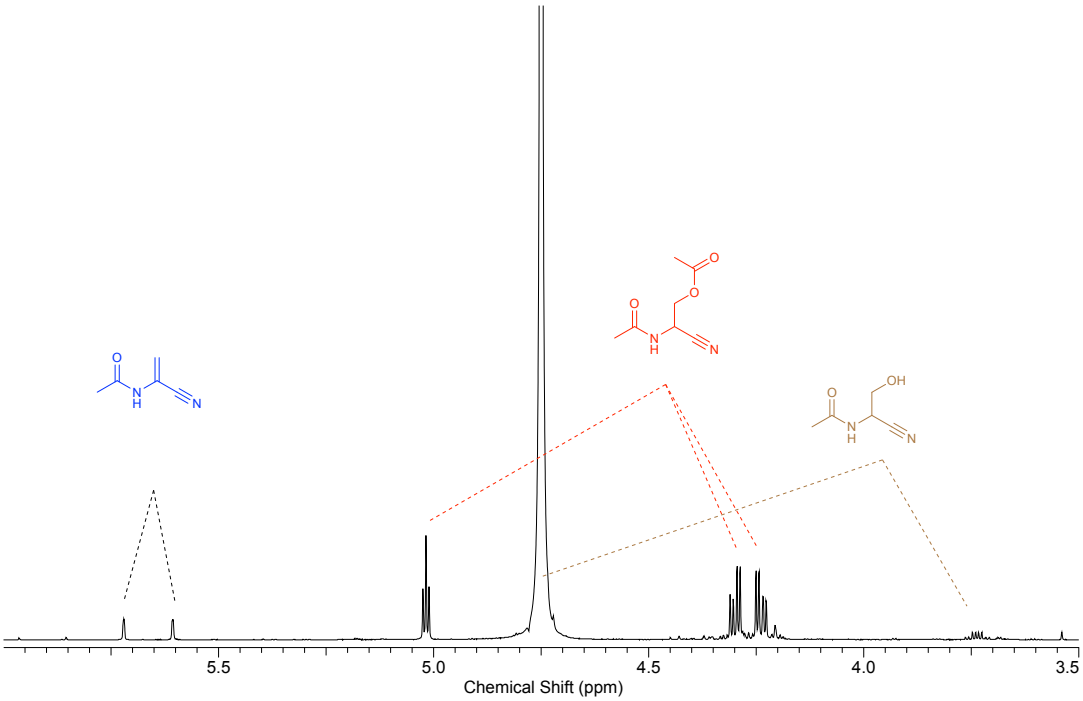


Fig. S9. ^{1}H NMR spectra (700 MHz, D_2O, 3.50–6.00 ppm) to show the reaction of **Ser-CN** (100 mM) with *N*-acetylimidazole **NAI** (500 mM) at pD 7 and room temperature after 19 h.

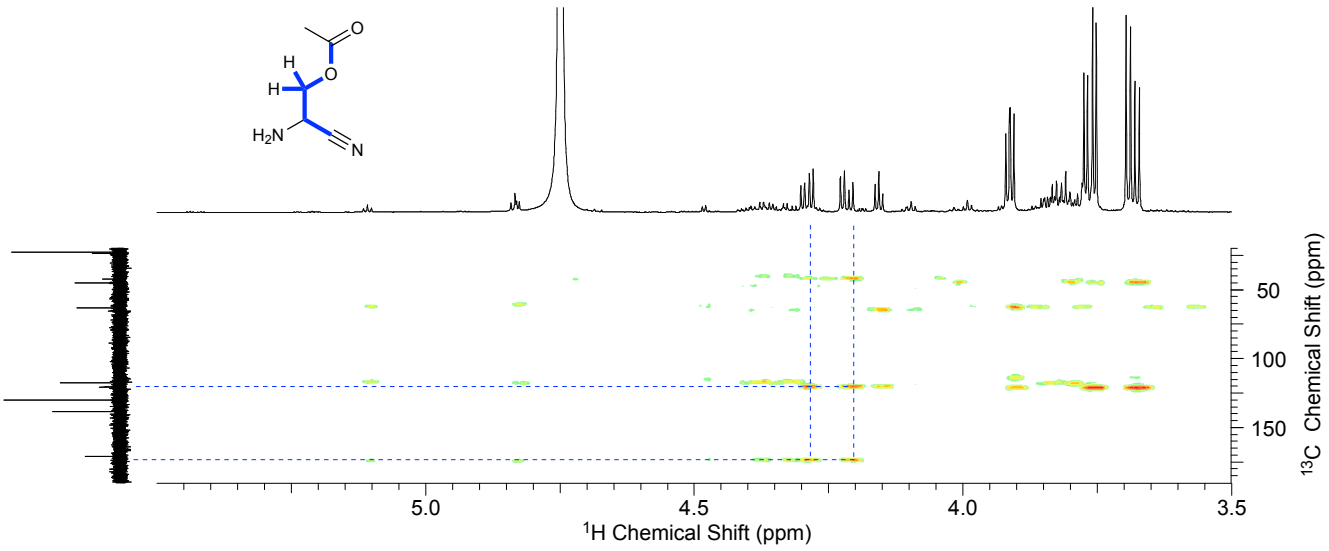
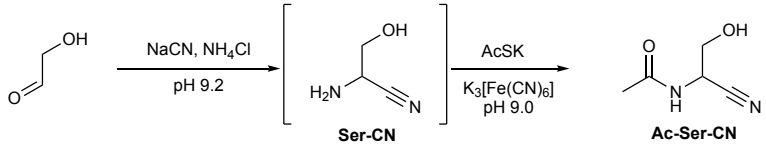


Fig. S10. ^{1}H–^{13}C HMBC (^{1}H–700 MHz [3.50–5.50 ppm], ^{13}C–176 MHz [20–190 ppm]) spectrum showing the diagnostic $^{3}J_{CH}$ coupling of (C3)–H and (C3)–H′ of **Ser^{Ac}-CN** at 4.29 and 4.22 ppm with ^{13}C resonances at 173.6 ppm (**C**OCH_3) and 120.6 ppm (C1), acquired after 5 min from the reaction of **Ser-CN** (100 mM) and *N*-acetylimidazole **NAI** (250 mM) at pD 7 and room temperature. Note that (C2)–H of **Ser-CN** has a $^{2}J_{CH}$ coupling to C1, but no $^{3}J_{CH}$ to **C**OCH_3. See Fig. S8a for the ^{1}H NMR spectrum.

Preparative synthesis and isolation of N-acetyl-DL-serine nitrile by oxidative acetylation with thioacetate



Glycolaldehyde **GC** (600 mg, 10 mmol), sodium cyanide (588 mg, 12 mmol) and ammonium chloride (2.65 g, 50 mmol) were dissolved in water (10 mL) and the solution was adjusted to pH 9.2 by addition HCl/NaOH. The solution was then stirred at room temperature. After 24 h the reaction was diluted with water (190 mL). Potassium thioacetate (3.42 g; 30 mmol) was added and the pH adjusted to 9.0 by addition of HCl/NaOH. Potassium hexacyanoferrate(III) (29.6 g; 90 mmol) was added, and the solution was stirred at room temperature for 20 min. The solution was adjusted to pH 9.0, centrifuged, and concentrated *in vacuo*. The residue was repeatedly triturated with methanol until **Ac-Ser-CN** was completely recovered from the solid. The methanolic extracts were concentrated *in vacuo*, and the residue purified by flash column chromatography (SiO_2; eluting with a gradient of petroleum ether/ethyl acetate 1:1 to 0:1). **Ac-Ser-CN** was obtained as a white solid (1.16 g, 91%). ^{1}H NMR (600 MHz, D_2O) δ_H 4.81 (1H, dd, J = 5.3, 5.3 Hz, H–(C2)), 3.80 (1H, ABX, J = 5.3, 11.7 Hz, H_a–(C3)), 3.76 (1H, ABX, J = 5.3, 11.7 Hz, H_b–(C3)),1.97 (3H, s, $COCH_3$). ^{13}C NMR (151 MHz, D_2O) δ 174.7 (**C**OCH_3), 118.3 (C1), 61.2 (C3), 43.7 (C2), 22.2 (CO**C**H_3). IR (cm^{-1}) 3260, 2938, 2240, 1618, 1526. HRMS-ESI $[M+H^+]^+$ calculated for formula $C_5H_9N_2O_2^+$, 129.0659; found 129.0660.

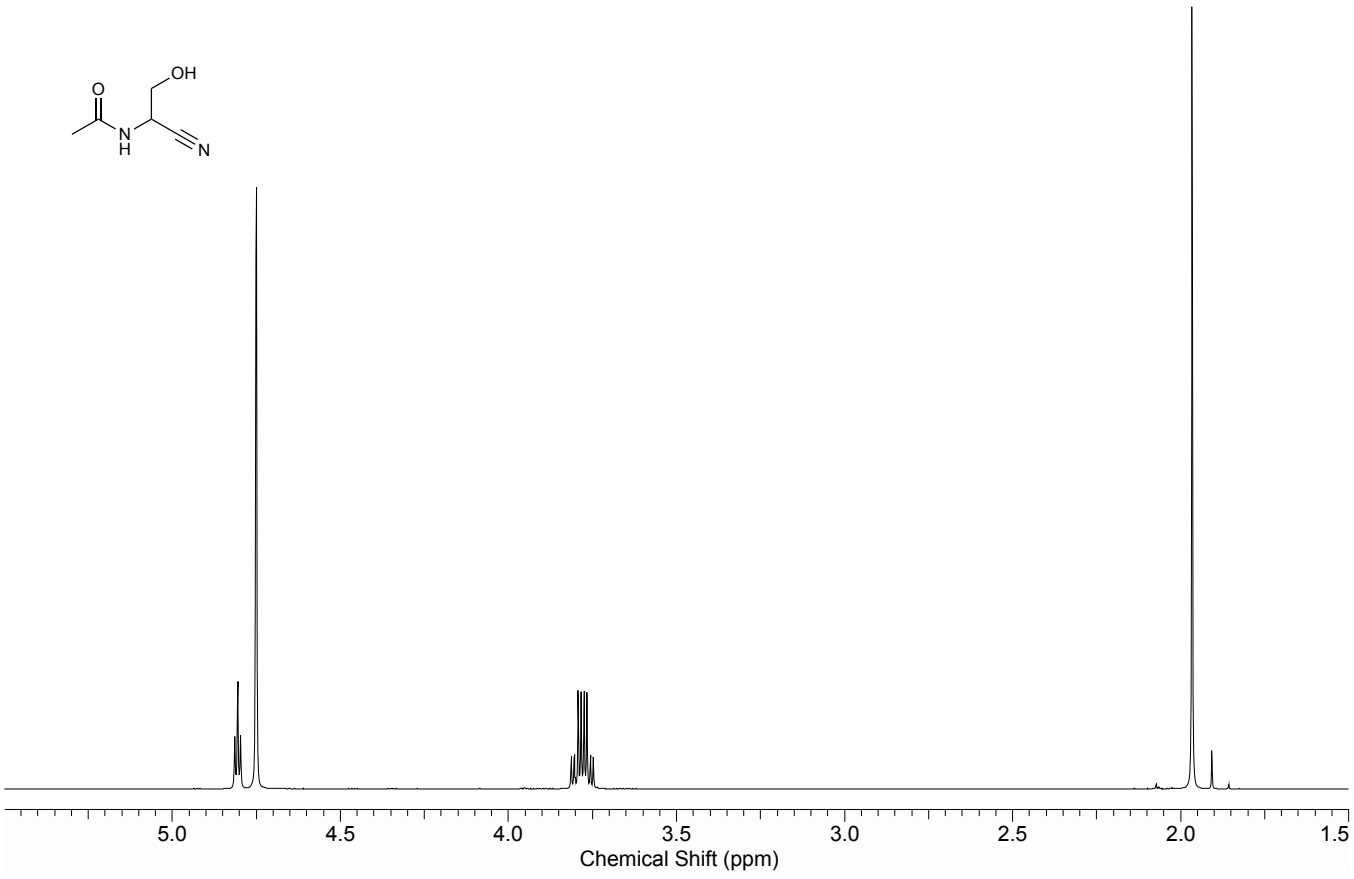


Fig. S11. ^{1}H NMR (600 MHz, D_2O, 1.50 – 5.50 ppm) spectrum to show **Ac-Ser-CN**.

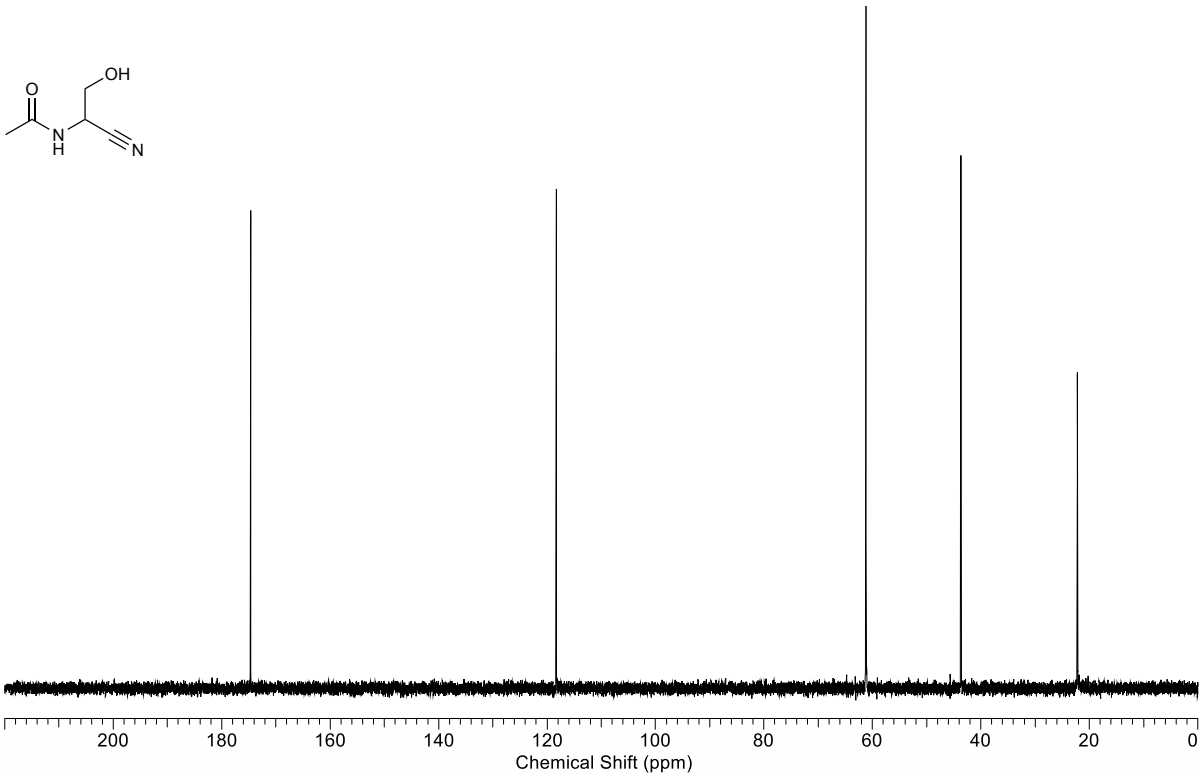
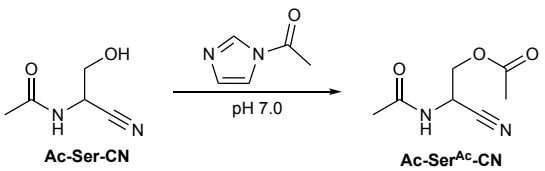


Fig. S12. ^{13}C NMR (151 MHz, D_2O, 0 – 220 ppm) spectrum to show **Ac-Ser-CN**.

Preparative synthesis and isolation of N,O-diacetyl-DL-serine nitrile by acetylation with N-acetylimidazole



Ac-Ser-CN **Ac-Ser^{Ac}-CN**

N-Acetyl-DL-serine nitrile **Ac-Ser-CN** (0.82 g, 6.38 mmol) and *N*-acetyl imidazole (2.81 g, 25.5 mmol) were dissolved in water (64 mL) at pH 7.0, incubated for 4 h and then lyophilised. The residue was then purified by flash column chromatography (SiO_2; eluting with petroleum ether/ethyl acetate 9:1 to 0:1) to yield *N,O*-diacetyl-DL-serine nitrile **Ac-Ser^{Ac}-CN** as a white solid (1.06 g, 98%). ^{1}H NMR (700 MHz, D_2O) δ_H 5.12 - 5.16 (1H, m, H-(C2)), 4.41 (1H, ABX, *J* = 11.2, 5.4 Hz, H_a-(C3)), 4.35 (1H, ABX, *J* = 11.2, 4.9 Hz, H_b-(C3)), 2.13 (3H, s, $OCOCH_3$), 2.02 (3H, s, $NCOCH_3$). ^{13}C NMR (176 MHz, D_2O) δ 174.5 (N<u>C</u>OCH_3), 173.8 (O<u>C</u>OCH_3), 117.4 (C1), 63.1 (C3), 40.7 (C2), 22.1 (NCO<u>C</u>H_3), 20.6 (OCO<u>C</u>H_3). IR (cm^{-1}) 3298, 3043, 2804, 2257, 1733, 1698, 1651. HRMS-ESI $[M+H^+]^+$ calculated for formula $C_7H_{11}N_2O_3^+$, 171.0764; found 171.0765.

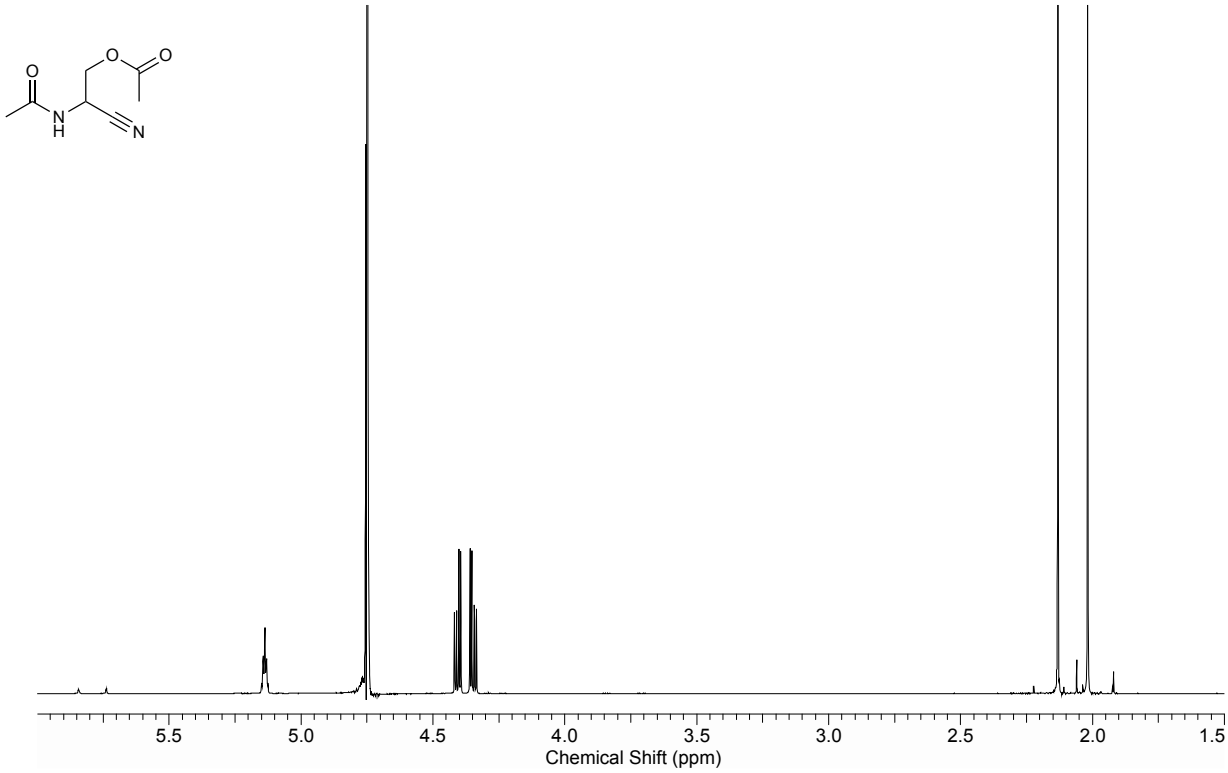


Fig. S13. ^{1}H NMR (700 MHz, H_2O, noesygppr1d, 1.50 – 5.00 ppm) spectrum to show **Ac-Ser^{Ac}-CN**.

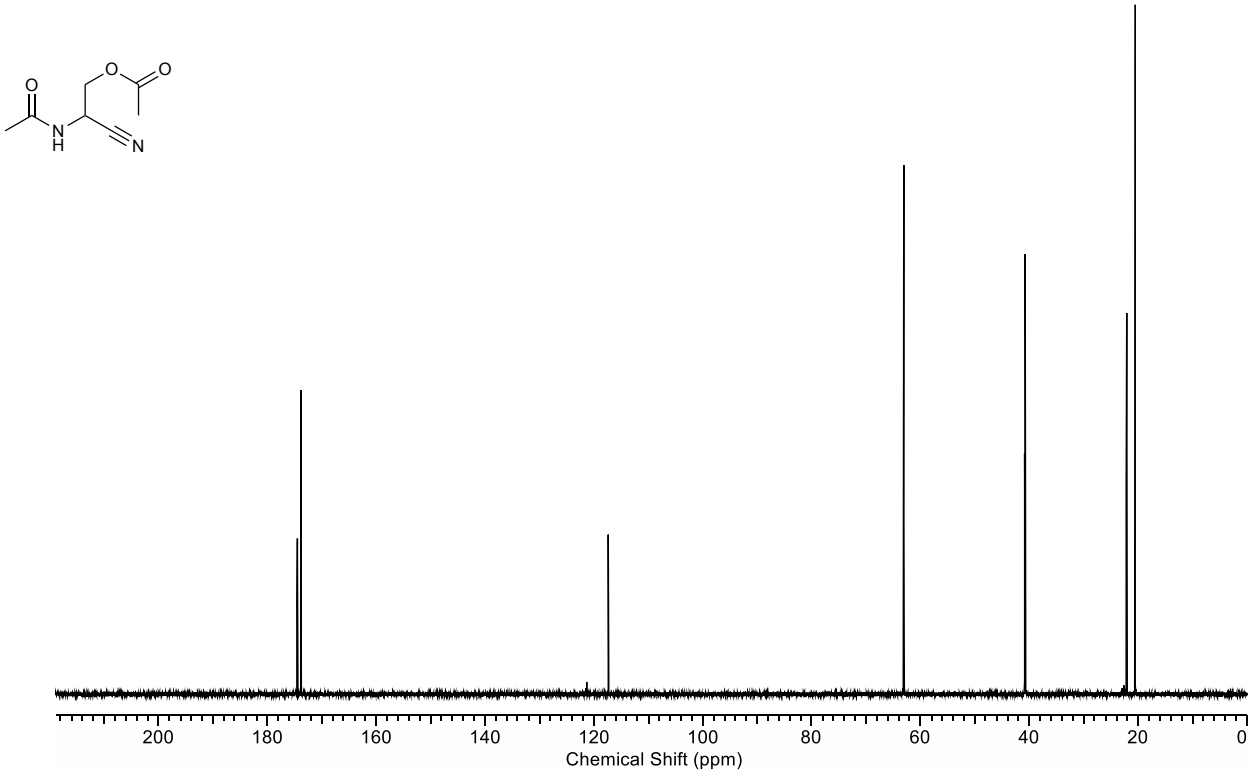
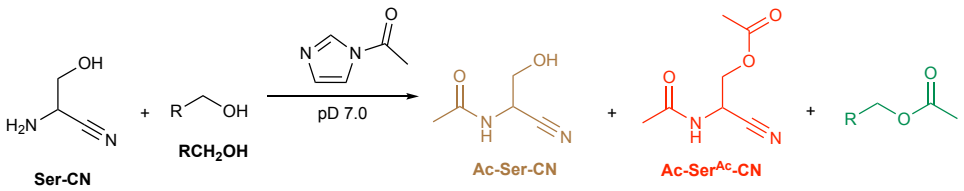


Fig. S14. ^{13}C NMR (176 MHz, H_2O, 0 – 220 ppm) spectrum to show **Ac-Ser^{Ac}-CN**.

Acetylation of DL-serine nitrile with N-acetylimidazole in competition with other alcohols



A solution of DL-serine nitrile **Ser-CN** (100 mM), an alcohol **R–CH_2OH** (100 mM) was adjusted to pD 7 with 4 M DCl/NaOD. *N*-Acetylimidazole **NAI** (5 equiv.) was added and the solution was readjusted to pD 7. The reaction was monitored by NMR spectroscopy (Fig. S15 – Fig. S17). Yields are reported in Table S4 after 6 h.

Competitor	Alcohol	-OH (%)	OAc (%)	Ser-CN:ROH
$HOCH_2CH_2OH$	Ser-CN	6	92	4
	ROH	70	28 (6)*	
$AcNHCH_2CH_2OH$	Ser-CN	7	92	3
	ROH	68	32	
$NCCH_2CH_2OH$	Ser-CN	7	91	1
	ROH	20	80	

Table S4. Yields of hydroxyl-acetylation of **Ser-CN** (100 mM) and an alcohol **RCH_2OH** (100 mM) after reaction with *N*-acetylimidazole **NAI** (5 equiv.) at pD 7 and room temperature. N.B. >98% **Ser-CN** was *N*-acetylated, and hydroxyl acetylation (Ser-OAc) yield are reported for **Ac-Ser^{Ac}-CN**. * 6% di-acetylated product (diacetylethylene glycol) was observed (Fig. S15).

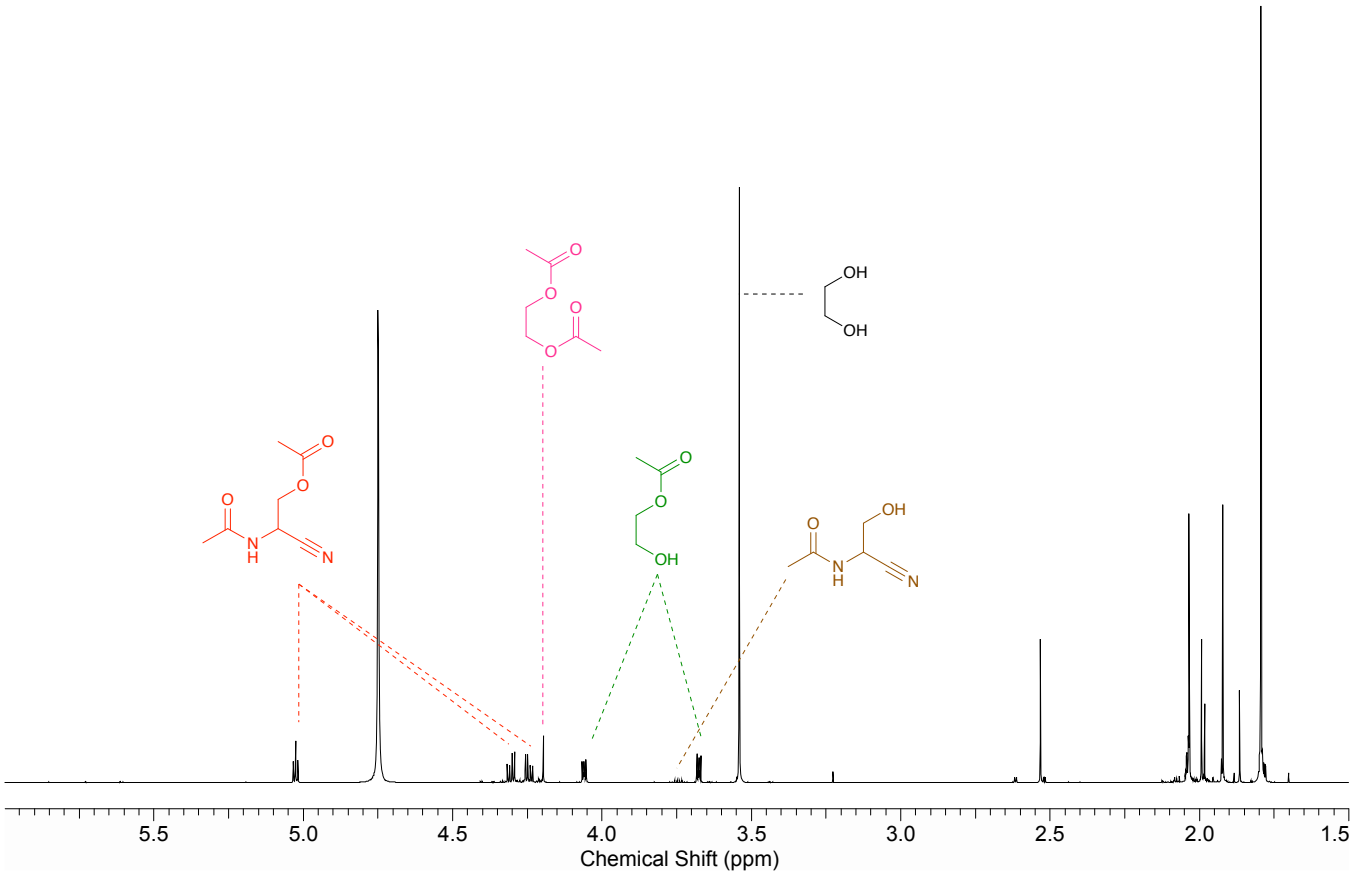


Fig. S15. ^{1}H NMR spectrum (700 MHz, D_2O, 1.50–6.00 ppm) to show the reaction of **Ser-CN** (100 mM) and ethylene glycol (100 mM) with *N*-acetylimidazole **NAI** (500 mM) at pD 7 and room temperature.

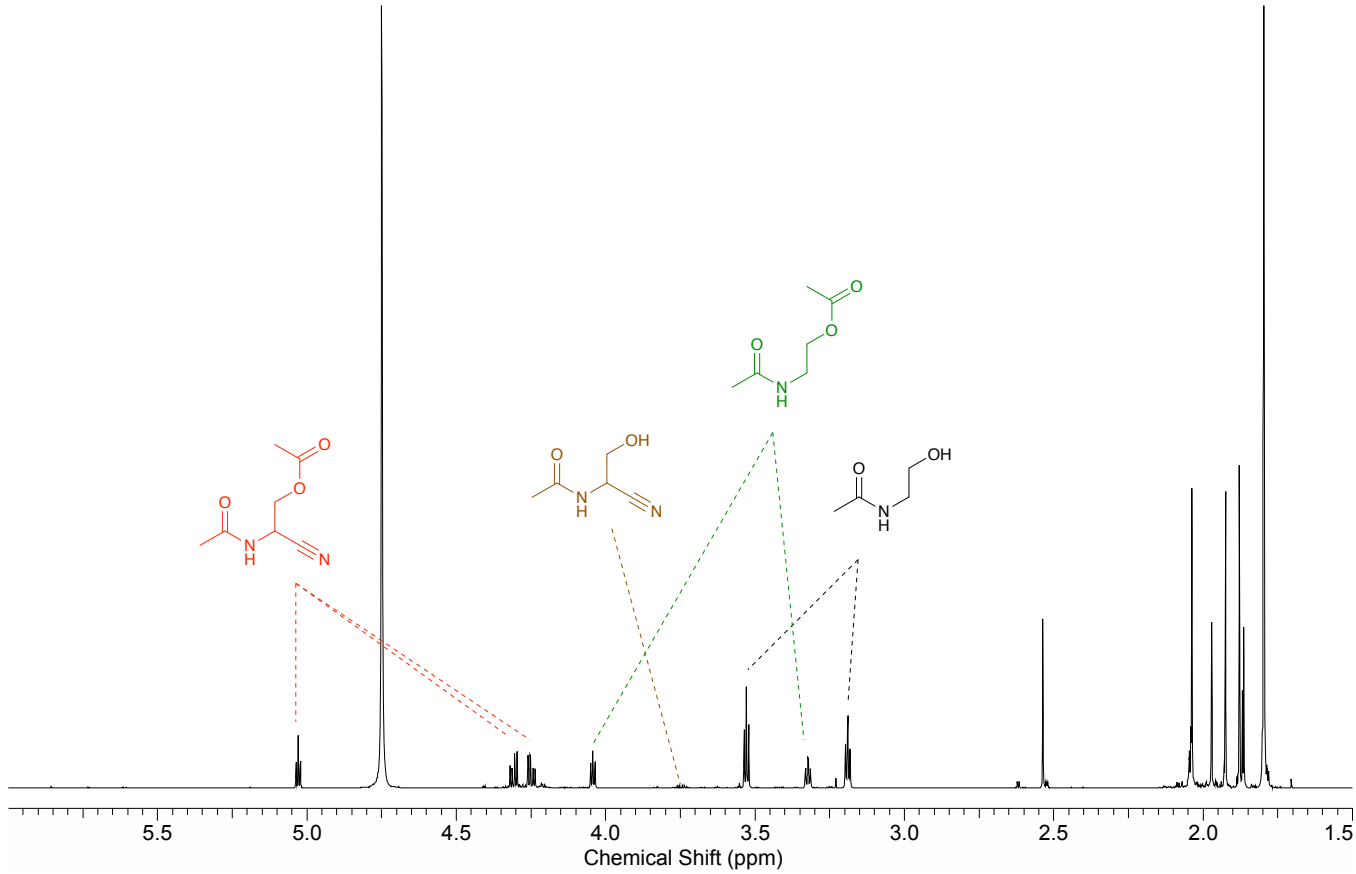


Fig. S16. ^{1}H NMR spectrum (700 MHz, D_2O, 1.50–6.00 ppm) to show the reaction of **Ser-CN** (100 mM) and *N*-acetylethanolamine (100 mM) with *N*-acetylimidazole **NAI** (500 mM) at pD 7 and room temperature.

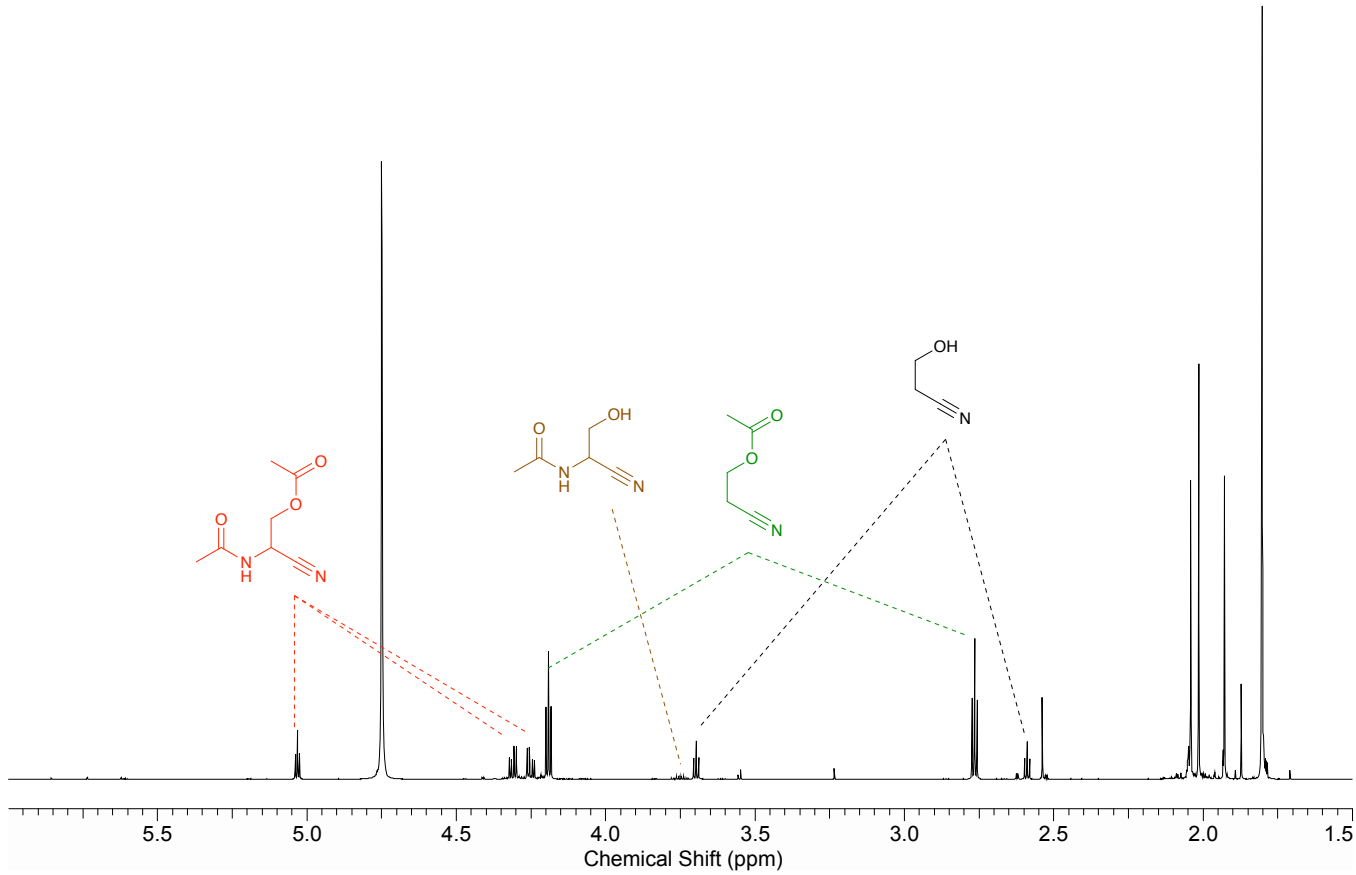
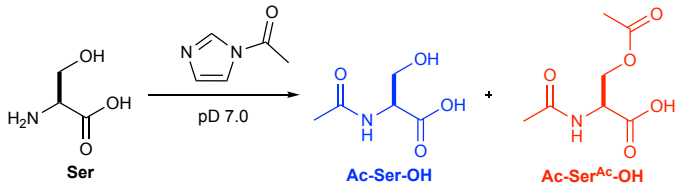


Fig. S17. ^{1}H NMR spectrum (700 MHz, D_2O, 1.50–6.00 ppm) to show the reaction of **Ser-CN** (100 mM) and 3-hydroxypropionitrile (100 mM) with *N*-acetylimidazole **NAI** (500 mM) at pD 7 and room temperature.

Acetylation of L-serine with N-acetylimidazole



A solution of L-serine **Ser** (100 mM) was adjusted to pD 7 with 4 M DCl/NaOD. *N*-Acetylimidazole **NAI** (5 equiv.) was added and the solution was readjusted to pD 7. The reaction was monitored by NMR spectroscopy, until the complete consumption of **NAI** was observed (5 h). *N*-Acetyl-L-serine **Ac-Ser-OH** was observed (88%) alongside *N,O*-diacetyl-L-serine **Ac-Ser^{Ac}-OH** (11%) (Fig. S18).

*Data for N-acetyl-L-serine **Ac-Ser-OH***

^{1}H NMR (700 MHz, D_2O) δ 4.16 (dd, *J* = 3.8, 6.3 Hz, 1H, (C2)–H), 3.74 (dd, *J* = 3.8, 11.6 Hz, 1H, (C3)–H), 3.69 (dd, *J* = 6.3, 11.6 Hz, 1H, (C3)–H′), 1.92 (s, 3H, $COCH_3$).

*Data for N,O-diacetyl-L-serine **Ac-Ser^{Ac}-OH***

^{1}H NMR (700 MHz, D_2O) δ 4.35 (dd, *J* = 3.8, 5.2 Hz, 1H, (C2)–H), 4.26 (dd, *J* = 5.2, 11.3 Hz, 1H, (C3)–H), 4.23 (dd, *J* = 3.8, 11.3 Hz, 1H, (C3)–H′), 1.95 (s, 3H, $COCH_3$), 1.91 (s, 3H, $COCH_3$).

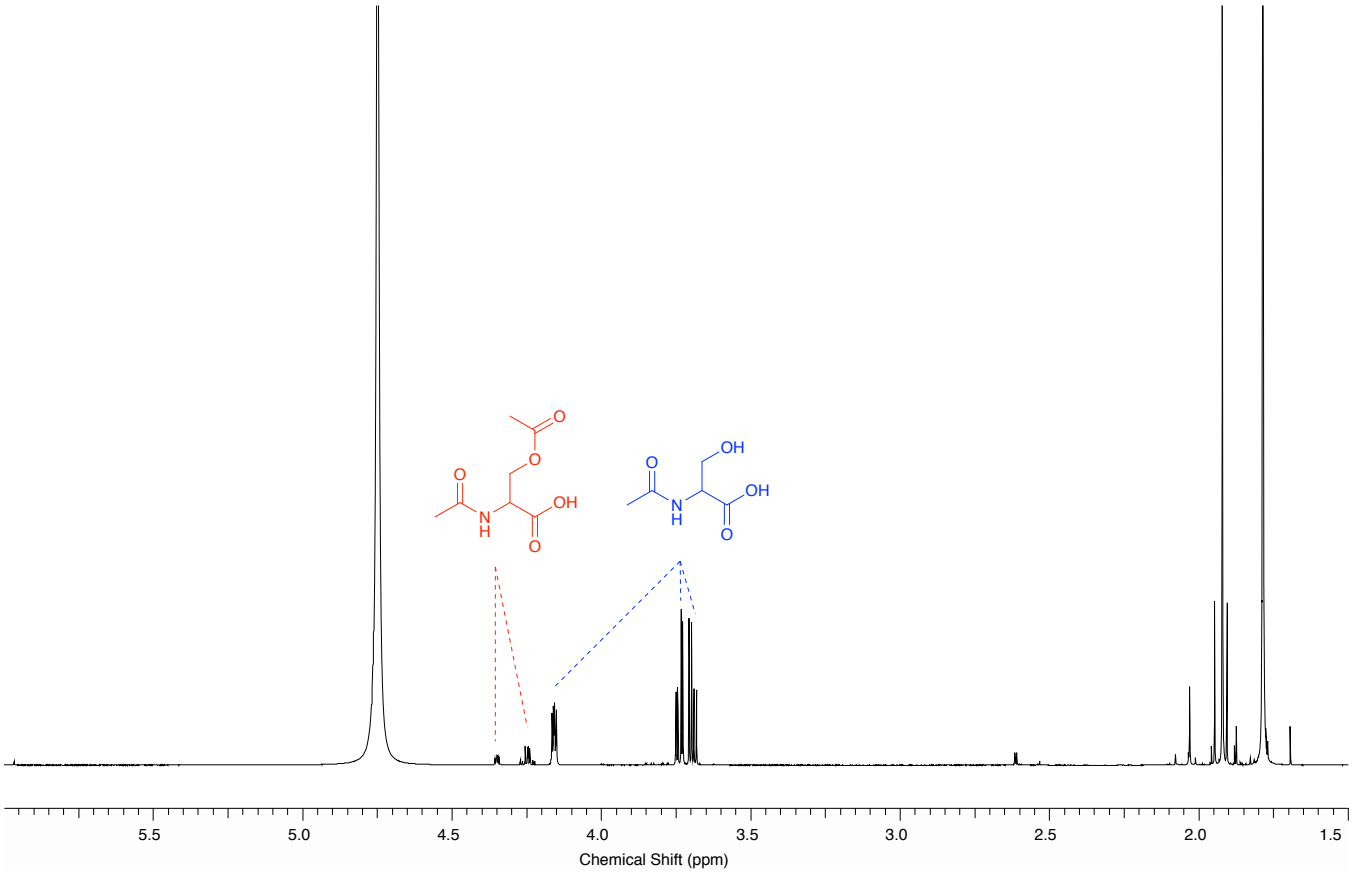
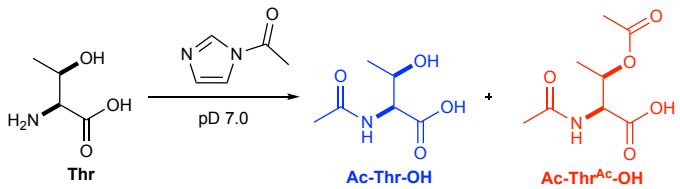


Fig. S18. ^{1}H NMR spectum (700 MHz, D_2O, 1.50–6.00 ppm) to show the reaction of **Ser** (100 mM) with *N*-acetylimidazole **NAI** (500 mM) at pD 7 and room temperature.

Acetylation of L-threonine with N-acetylimidazole



A solution of L-threonine **Thr** (100 mM) was adjusted to pD 7 with 4 M DCl/NaOD. *N*-Acetylimidazole **NAI** (5 equiv.) was added and the solution was readjusted to pD 7. The reaction was monitored by NMR spectroscopy, until the complete consumption of **NAI** was observed (5 h). *N*-Acetyl-L-threonine **Ac-Thr-OH** (>95%) was observed (Fig. S19) alongside trace amounts of *N,O*-diacetyl-L-threonine nitrile **Ac-Thr^{Ac}-OH**.

Data for N-acetyl-L-threonine ***Ac-Thr-OH***

^{1}H NMR (700 MHz, D_2O) δ 4.11 (dq, *J* = 4.0, 6.5 Hz, 1H, (C3)–H), 4.01 (d, *J* = 4.0 Hz, 1H, (C2)–H), 1.93 (s, 3H, $COCH_3$), 1.04 (d, *J* = 6.5 Hz, 3H, (C4)–H_3).

Data for N,O-diacetyl-L-threonine ***Ac-Thr^{Ac}-OH*** *(partial assignment).*

^{1}H NMR (700 MHz, D_2O) δ 5.25 (dq, *J* = 3.2, 6.4 Hz, 1H, (C3)–H), 4.22 (d, *J* = 3.2 Hz, 1H, (C2)–H).

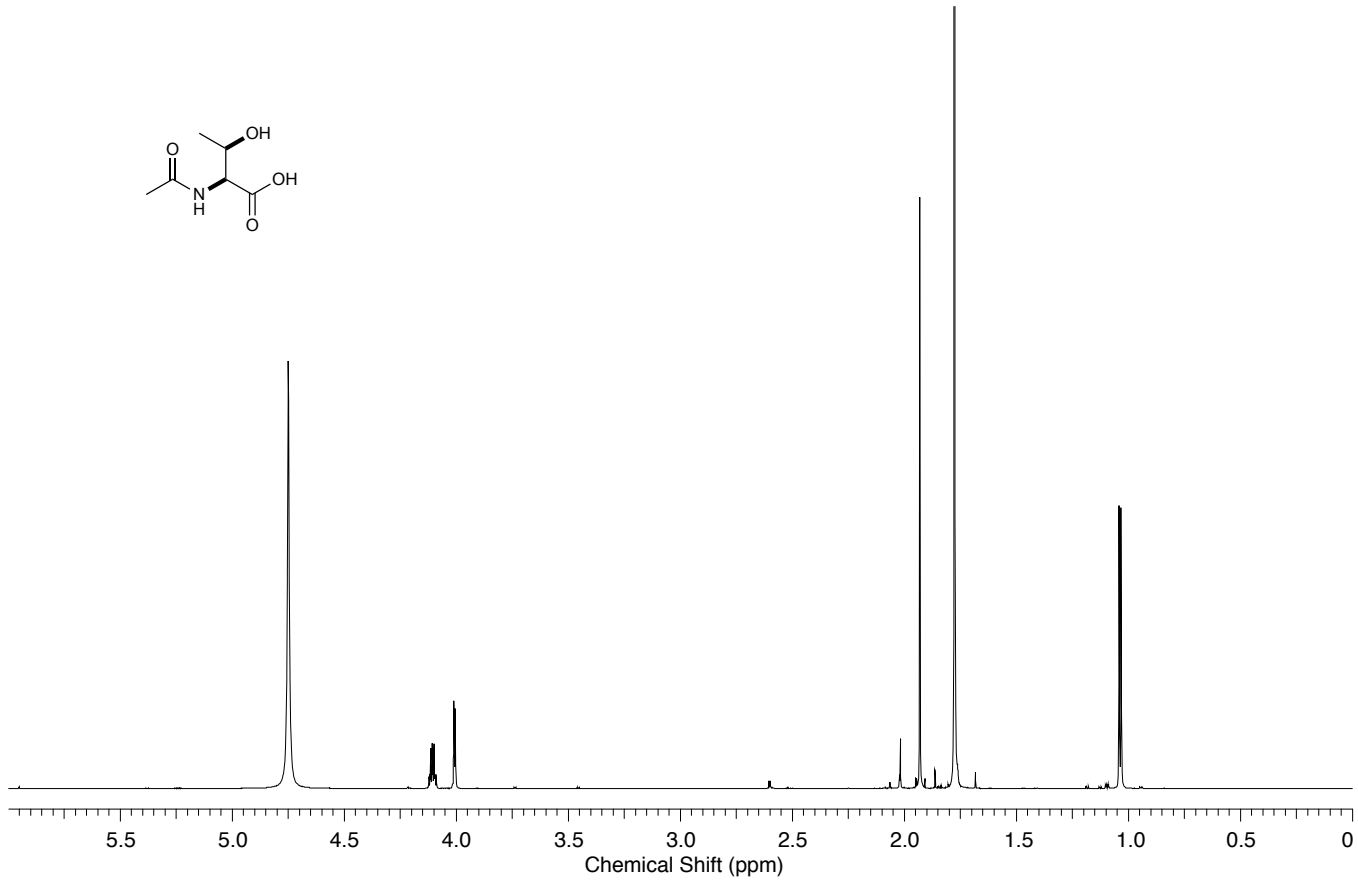
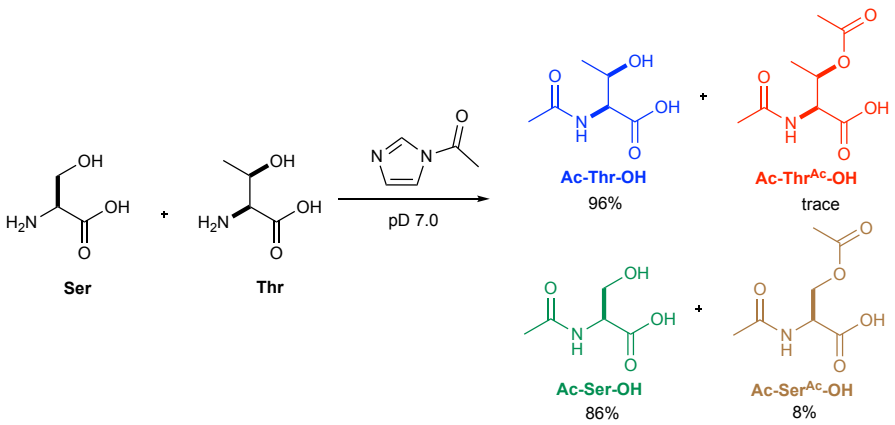


Fig. S19. ^{1}H NMR (700 MHz, D_2O, 0.00 – 5.00 ppm) spectrum to show the reaction of L-threonine (**Thr**; 100 mM) with *N*-acetylimidazole (**NAI**; 5 equiv.) after 5 h at pD 7 and room temperature.

Acetylation of L-serine and L-threonine with N-acetylimidazole



A solution of L-serine **Ser** and L-threonine **Thr** (100 mM) was adjusted to pD 7 with 4 M DCl/NaOD. *N*-Acetylimidazole **NAI** (5 equiv.) was added and the solution was readjusted to pD 7. The reaction was monitored by NMR spectroscopy, until the complete consumption of **NAI** was observed (5 h). **Thr** underwent conversion to **Ac-Thr-OH** (>95%), with **Ac-Thr^{Ac}-OH** detected in only trace amounts. **Ser** underwent conversion to a mixture of **Ac-Ser-OH** (86%) and **Ac-Ser^{Ac}-OH** (6%) (Fig. S20).

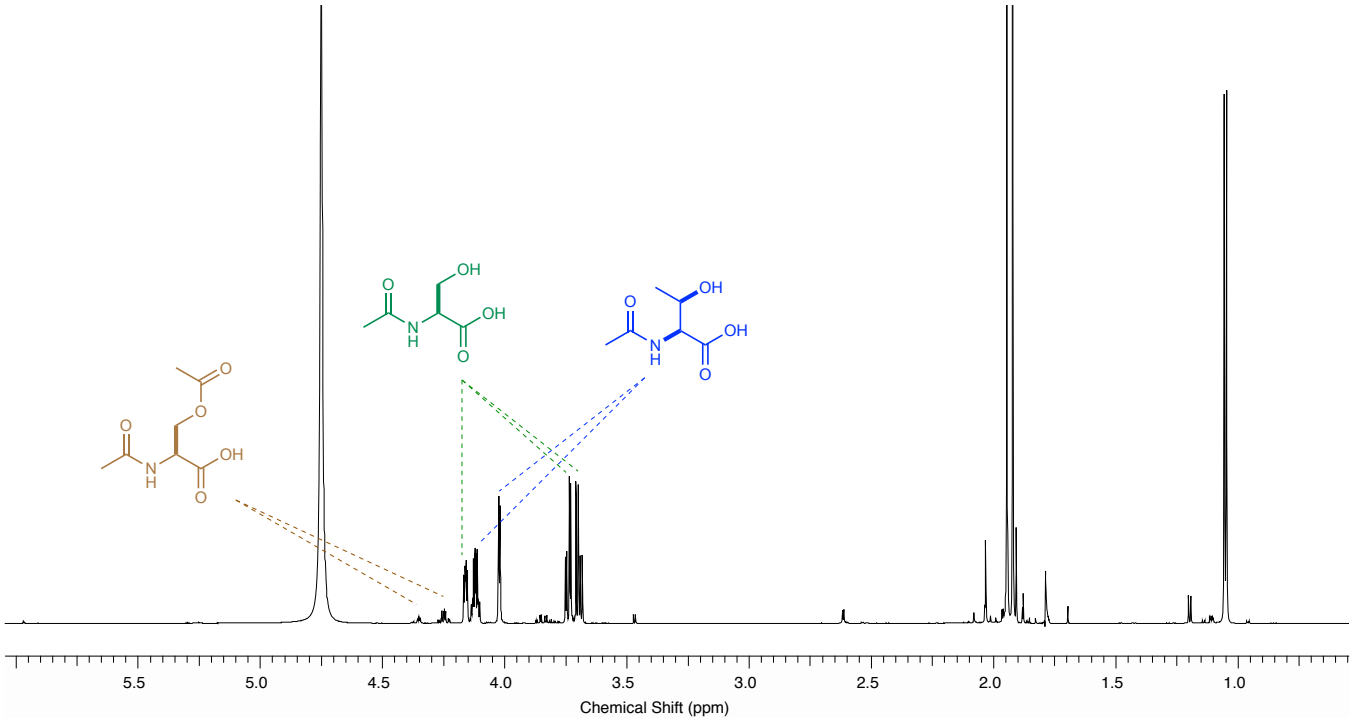
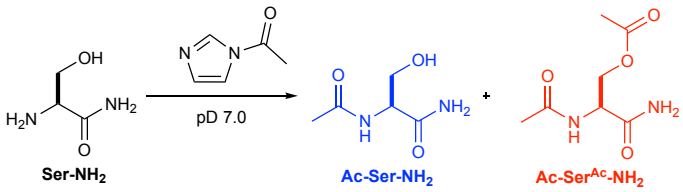


Fig. S20. ^{1}H NMR (700 MHz, D_2O, 0.50 – 6.00 ppm) spectrum to show the reaction of L-threonine (**Thr**; 100 mM) and L-serine (**Ser**; 100 mM) with *N*-acetylimidazole (**NAI**; 5 equiv.) after 5 h at pD 7 and room temperature.

Acetylation of L-serinamide with N-acetylimidazole



A solution of L-serinamide **Ser-NH₂** (100 mM) was adjusted to pD 7 with 4 M DCl/NaOD. *N*-Acetylimidazole **NAI** (5 equiv.) was added and the solution was readjusted to pD 7. The reaction was monitored by NMR spectroscopy, until the complete consumption of **NAI** was observed. *N,O*-Diacetyl-L-serinamide **Ac-Ser^{Ac}-NH₂** (67%) and *N*-acetyl-L-serinamide (33%) were observed after 5 h.

Data for N-acetyl-L-serinamide ***Ac-Ser-NH₂***

^{1}H NMR (600 MHz, D_2O) δ 4.20 (dd, *J* = 4.6, 5.1 Hz, 1H, (C2)–H), 3.70 (dd, *J* = 5.1, 11.7 Hz, 1H, (C3)–H), 3.66 (dd, *J* = 4.6, 11.7 Hz, 1H, (C3)–H′), 1.89 (s, 3H, $COCH_3$).

Data for N,O-diacetyl-L-serinamide ***Ac-Ser^{Ac}-NH₂***

^{1}H NMR (600 MHz, D_2O) δ 4.44 (dd, *J* = 4.2, 5.1 Hz, 1H, (C2)–H), 4.27 (dd, *J* = 5.1, 11.6 Hz, 1H, (C3)–H), 4.15 (dd, *J* = 4.2, 11.6 Hz, 1H, (C3)–H′), 1.92 (s, 3H, $COCH_3$), 1.88 (s, 3H, $COCH_3$).

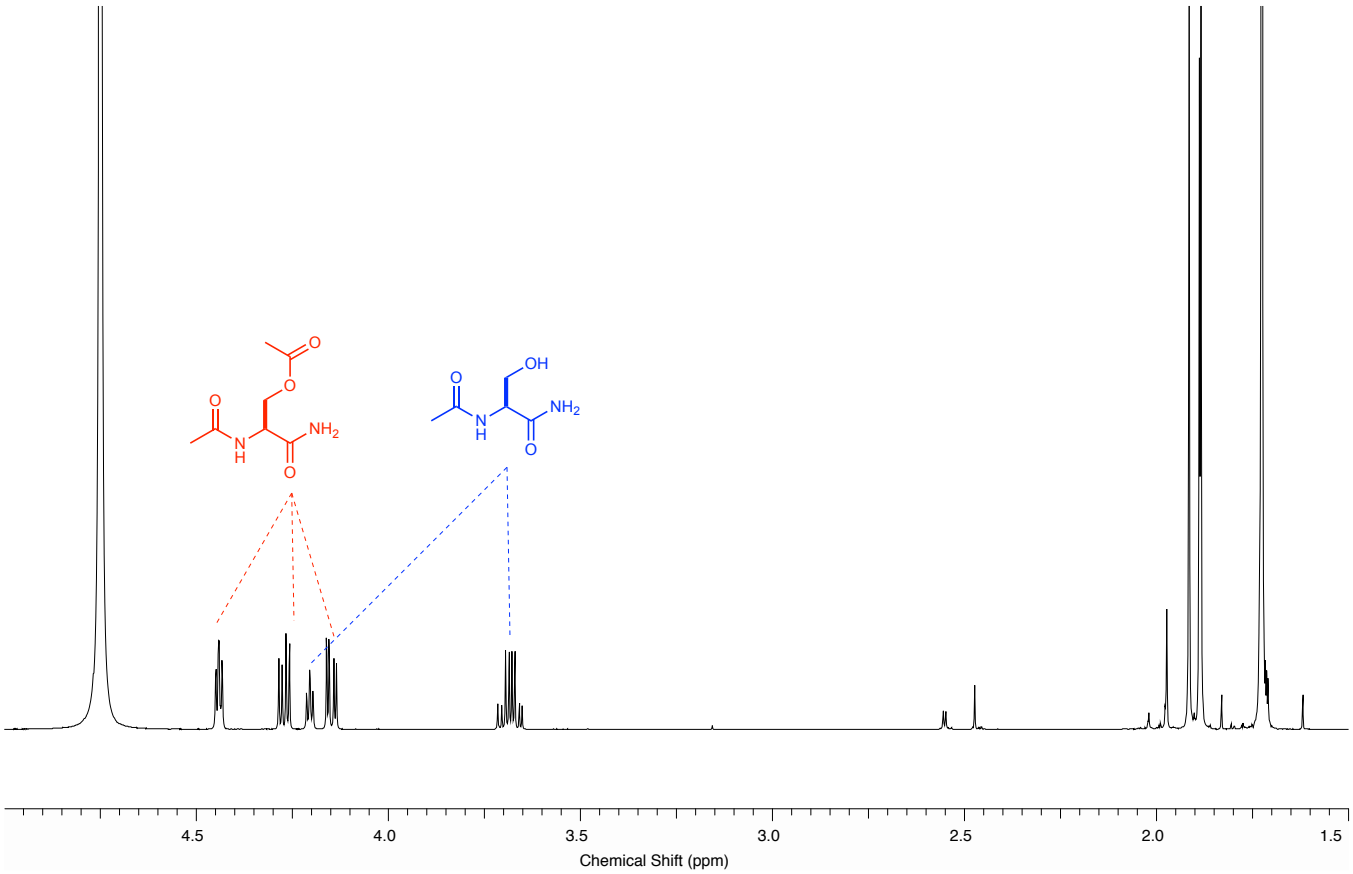
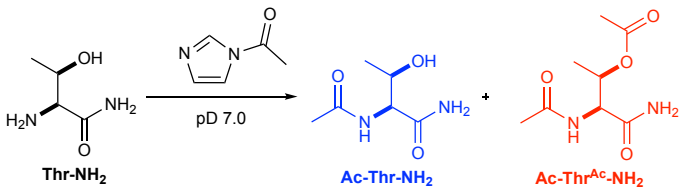


Fig. S21. ^{1}H NMR spectum (600 MHz, D_2O, 1.50–5.50 ppm) to show the reaction of **Ser-NH₂** (100 mM) with *N*-acetylimidazole **NAI** (500 mM) at pD 7 and room temperature.

Acetylation of L-threoninamide with N-acetylimidazole



A solution of L-threoninamide **Thr-NH₂** (100 mM) was adjusted to pD 7 with 4 M DCl/NaOD. *N*-Acetylimidazole **NAI** (5 equiv.) was added and the solution was readjusted to pD 7. The reaction was monitored by NMR spectroscopy, until the complete consumption of **NAI** was observed. *N,O*-Diacetyl-L-threoninamide **Ac-Thr^{Ac}-NH₂** (<5%) and *N*-acetyl-L-threoninamide (95%) were observed after 5 h (Fig. S22).

*Data for N-acetyl-L-threoninamide **Ac-Thr-NH₂***

^{1}H NMR (600 MHz, D_2O) δ 4.11 - 4.05 (m, 2H, (C2)–H, (C3)–H), 1.91 (s, 3H, $COCH_3$), 1.02 (d, *J* = 6.3 Hz, 3H, (C4)–H_3).

*Data for N,O-diacetyl-L-threoninamide **Ac-Thr^{Ac}-NH₂***

^{1}H NMR (600 MHz, D_2O) δ 5.22 (dq, *J* = 2.9, 6.5 Hz, 1H, (C3)–H), 4.32 (d, *J* = 2.0 Hz, 1H, (C2)–H), 1.93 (s, 3H, $COCH_3$), 1.88 (s, 3H, $COCH_3$), 1.08 (d, *J* = 6.5 Hz, 3H, (C4)–H_3).

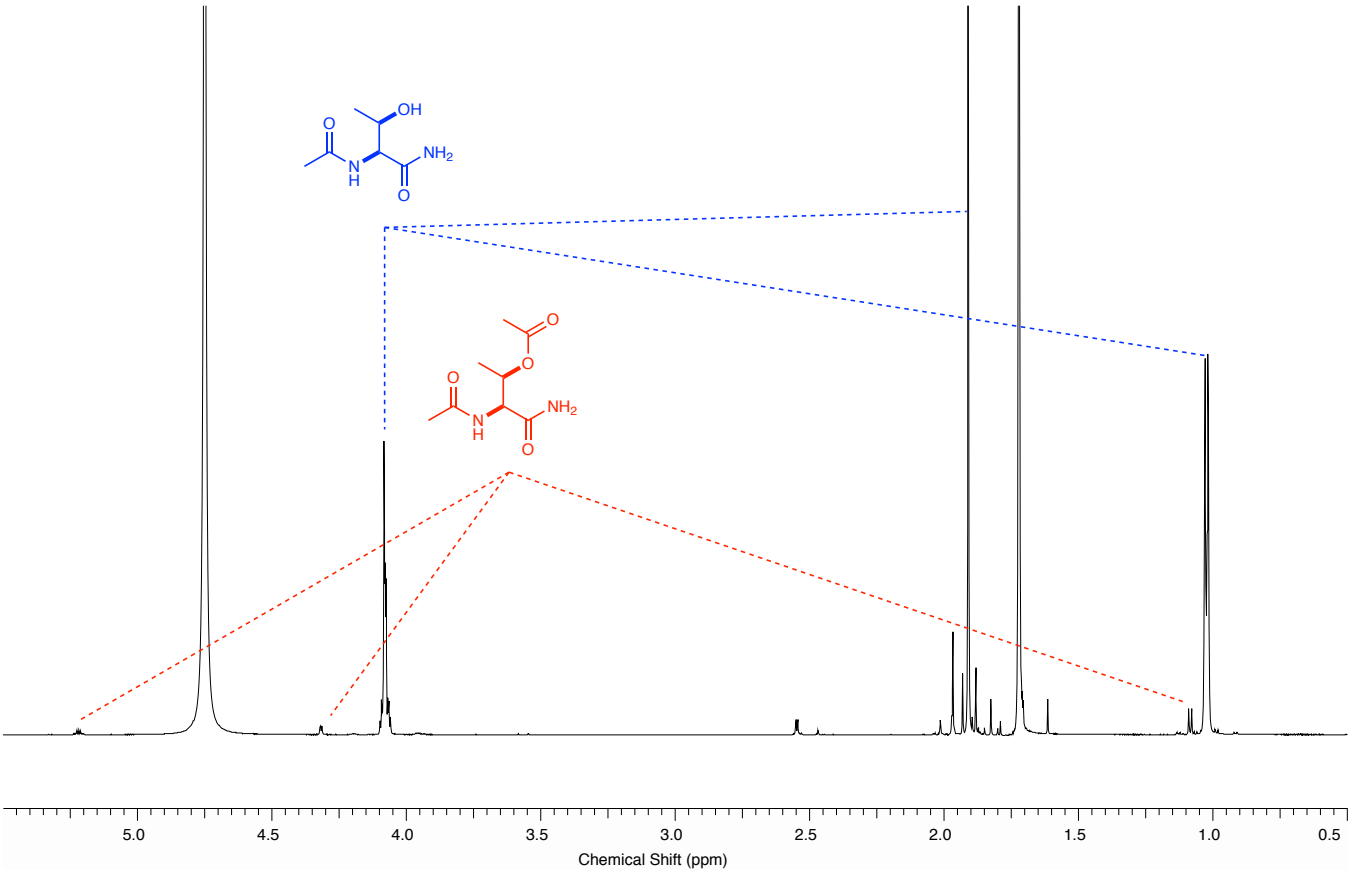
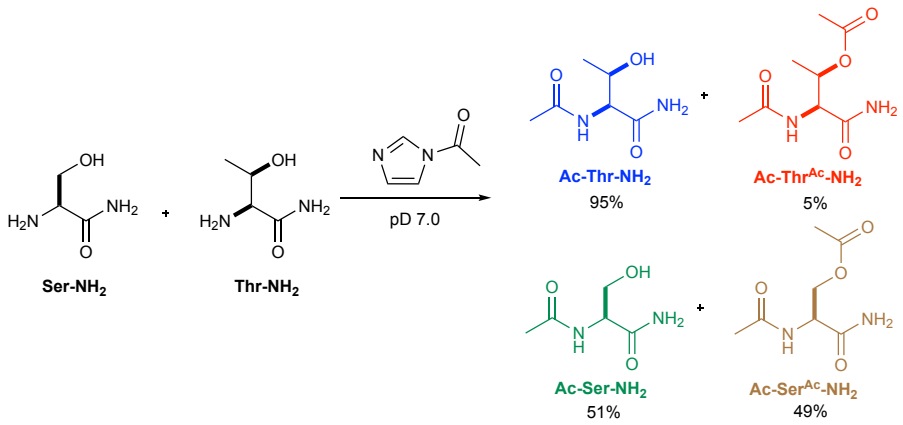


Fig. S22. ^{1}H NMR spectum (600 MHz, D_2O, 0.50–5.50 ppm) to show the reaction of **Thr-NH₂** (100 mM) with *N*-acetylimidazole **NAI** (500 mM) at pD 7 and room temperature.

Acetylation of L-serinamide and L-threoninamide with N-acetylimidazole



A solution of L-serinamide **Ser-NH$_2$** and L-threoninamide **Thr-NH$_2$** (100 mM) was adjusted to pD 7 with 4 M DCl/NaOD. *N*-Acetylimidazole **NAI** (5 equiv.) was added and the solution was readjusted to pD 7. The reaction was monitored by NMR spectroscopy, until the complete consumption of **NAI** was observed (5 h). **Thr-NH$_2$** underwent conversion to **Ac-Thr-OH** (95%) and **Ac-Thr^{Ac}-NH$_2$** (5%). **Ser-NH$_2$** underwent conversion to a mixture of **Ac-Ser-NH$_2$** (51%) and **Ac-Ser^{Ac}-NH$_2$** (49%).

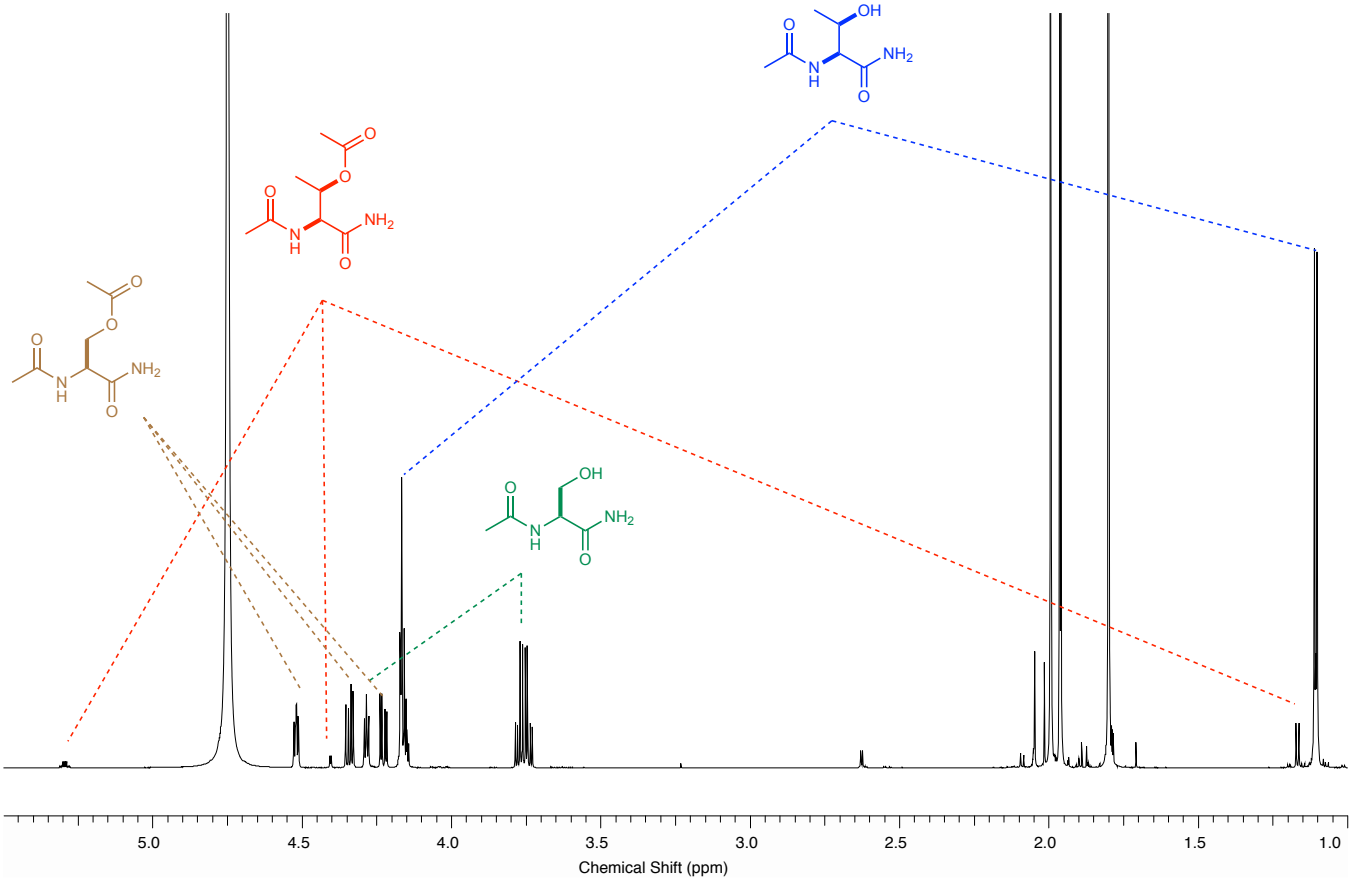
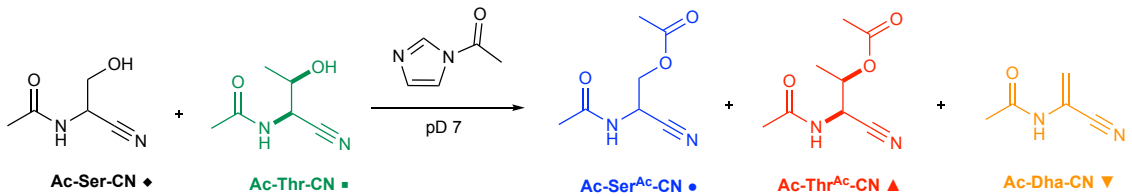


Fig. S23. ^{1}H NMR (700 MHz, D$_2$O, 1.50 – 5.50 ppm) spectrum to show the reaction of L-threoninamide (**Thr-NH$_2$**; 100 mM) and L-serinamide (**Ser-NH$_2$**; 100 mM) with *N*-acetylimidazole (**NAI**; 5 equiv.) after 5 h at pD 7 and room temperature

Acetylation of N-acetyl-DL-serine nitrile and N-acetyl-L-threonine nitrile with N-acetylimidazole



A solution of *N*-acetyl-DL-serine nitrile **Ac-Ser-CN** (100 mM) and *N*-acetyl-L-threonine nitrile **Ac-Thr-CN** (100 mM) was adjusted to pD 7 with 4 M DCl/NaOD. *N*-Acetylimidazole **NAI** (1 equiv.) was added and the solution was readjusted to pD 7. The reaction was monitored by NMR spectroscopy, until the complete consumption of **NAI** was observed (1 h). **Ac-Thr-CN** underwent conversion to **Ac-Thr^{Ac}-OH** (10%). **Ac-Ser-CN** underwent conversion to **Ac-Ser^{Ac}-CN** (65%) and trace amounts of **Ac-Dha-CN** (Fig. S24).

Data for N-acetyl-DL-serine nitrile ***Ac-Ser-CN***

^{1}H NMR (700 MHz, D_2O, noesygppr1d) δ 4.85 (dd, *J* = 5.4, 5.4 Hz, 1H, (C2)–H), 3.85 (ABX, *J* = 5.4, 11.7 Hz, 1H, (C3)–H), 3.82 (ABX, *J* = 5.4, 11.7 Hz, 1H, (C3)–H′), 2.02 - 2.01 (m, 3H, $COCH_3$).

Data for N,O-diacetyl-DL-serine nitrile ***Ac-Ser^{Ac}-CN***

^{1}H NMR (700 MHz, D_2O, noesygppr1d) δ 5.13 (dd, *J* = 5.2, 5.2 Hz, 1H, (C2)–H), 4.40 (ABX, *J* = 5.2, 11.4 Hz, 1H, (C3)–H), 4.34 (ABX, *J* = 5.2, 11.4 Hz, 1H, (C3)–H′), 2.13 (m, 3H, $COCH_3$), 2.01 (s, 3H, $COCH_3$).

Data for N-acetyl-L-threonine nitrile ***Ac-Thr-CN***

^{1}H NMR (700 MHz, D_2O, noesygppr1d) δ 4.83 (d, *J* = 4.9 Hz, 1H, (C2)–H), 4.13 (dq, *J* = 4.9, 6.4 Hz, 1H, (C3)–H), 2.03 (s, 3H, $COCH_3$), 1.25 (d, *J* = 6.4 Hz, 3H, (C4)–H_3).

Data for N,O-diacetyl-L-threonine nitrile ***Ac-Thr^{Ac}-CN*** *(partial assignment)*

^{1}H NMR (700 MHz, D_2O, noesygppr1d) δ 5.23 (dq, *J* = 4.7, 6.4 Hz, 1H, (C3)–H), 5.07 (d, *J* = 4.7 Hz, 1H, (C2)–H), 2.11 (s, 3H, $COCH_3$), 1.30 (d, *J* = 6.4 Hz, 3H, (C4)–H_3).

Data for N-acetyldehydroalanine nitrile ***Ac-Dha-CN***

^{1}H NMR (700 MHz, D_2O, noesygppr1d) δ 5.83 (d, *J* = 1.8 Hz, 1H, (C3)–H), 5.72 (d, *J* = 1.8 Hz, 1H, (C3)–H′).

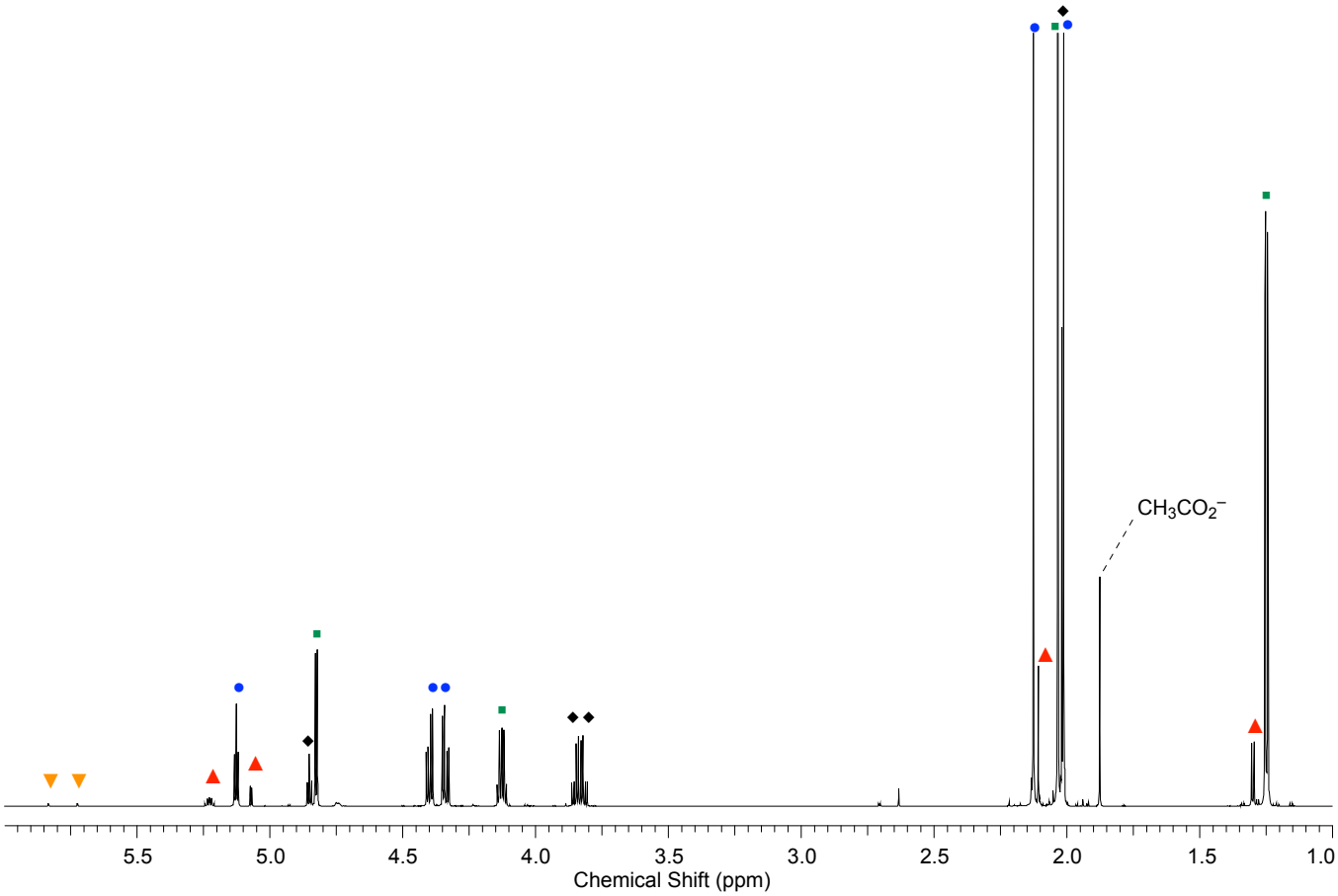
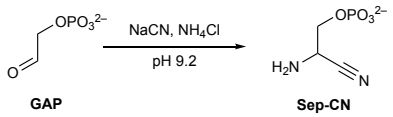


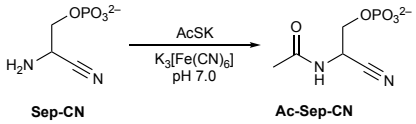
Fig. S24. ^{1}H NMR (700 MHz, D_2O, noesygppr1d,, 1.00 – 6.00 ppm) spectrum to show the reaction of *N*-acetyl-DL-serine nitrile (**Ac-Ser-CN**; 100 mM) and *N*-acetyl-L-threonine nitrile (**Ac-Thr-CN**; 100 mM) with *N*-acetylimidazole (**NAI**; 100 mM) after 1 h at pD 7 and room temperature.

Synthesis DL-phosphoserine nitrile



Glycolaldehyde phosphate **GAP** (200 mM), sodium cyanide (3 equiv.) and ammonium chloride (5 equiv.) were dissolved in water and the solution was adjusted to pH 9.5 by addition of HCl/NaOH. The reaction was then was monitored by periodic acquisition of NMR spectra (Fig. S25a). DL-Phosphoserine nitrile **Sep-CN** was used without further purification. ^{1}H NMR (700 MHz, H_2O) δ 4.06 (1H, app. t, *J* = 5.4 Hz, H-(C2)), 3.90 (1H, ABXY, *J* = 10.5, 5.8, 5.4 Hz, H_a-(C3)), 3.86 (1H, ABXY, *J* =, 10.5, 5.8, 5.4 Hz, 1 H). ^{13}C NMR (176 MHz, H_2O) δ 122.0 (C1), 65.4 (d, *J* = 4.0 Hz, C3), 44.5 (d, *J* = 8.4 Hz, C2). ^{31}P NMR (284 MHz, H_2O ^{1}H decoupled) δ 4.0. (*33*)

Acetylation of DL-phosphoserine nitrile



A solution of DL-phosphoserine nitrile **Sep-CN** (100 mM, pH 7.0) was sparged with argon for 15 minutes before potassium hexacyanoferrate(III) ($K_3[Fe(CN)_6]$;10 equiv.) and potassium thioacetate (5 equiv.) were added. The solution was stabilised at pH 7.0 by addition of HCl/NaOH and then incubated at room temperature. After 1 h the reaction was centrifuged and the supernatant analysed by NMR spectroscopy (Fig. S25b). The formation of **Ac-Sep-CN** was observed in 80% yield.

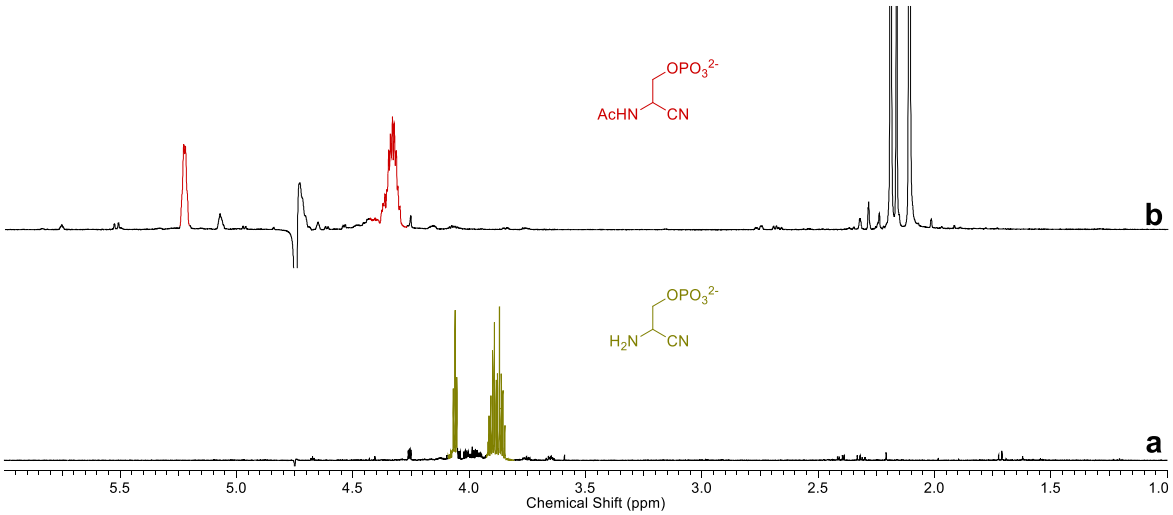
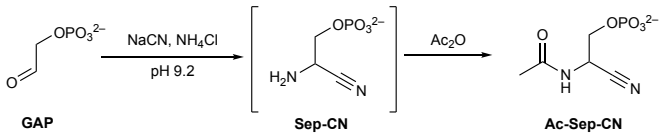


Fig. S25. ^{1}H NMR spectra (700 MHz, H_2O, noesygppr1d, 1.00–6.00 ppm) to show: **a**) the formation of **Sep-CN** from the reaction of glycolaldehyde phosphate **GAP** (200 mM), sodium cyanide (1.2 equiv.) and ammonia (5 equiv.) at room temperature and pH 9.5 after 6 d; **b**) the reaction of **Sep-CN** (100 mM) with potassium thioacetate (5 equiv.) and potassium hexacyanoferrate(III) ($K_3[Fe(CN)_6]$;10 equiv.) at pH 7.0.

Preparative synthesis and isolation of N-acetyl-phospho-DL-serine nitrile



GAP **Sep-CN** **Ac-Sep-CN**

Glycolaldehyde phosphate **GAP** (200 mM, 1 mmol), sodium cyanide (3 equiv.) and ammonium chloride (5 equiv.) were dissolved in water at pH 9.5 and stirred for 6 d. The solution was then cooled to 0 °C and acetic anhydride (25 equiv.) was added. The resulting solution was stirred for 20 min at 0 °C, before a second batch of acetic anhydride (12 equiv.) was added. The solution was then stirred at 0 °C for 30 min. The solution was then warmed to room temperature and lyophilised. The residue was the purified by ion exchange chromatography (formate (CO_2H^-) form, prepared from Dowex® 50W×8 ion-exchange resin (200-400 mesh, Cl^- form), eluting with a 0.1 M to 1 M gradient of ammonium formate at pH 4.0). The fractions that contained **Ac-Sep-CN** (0.4 M and 0.5 M formate) were lyophilised to give a white solid (0.43 mmol, 43%). ^{1}H NMR (700 MHz, H_2O) δ 5.02–4.93 (1H, m, H-(C2)), 4.11–4.06 (1H, m, H_a-(C3)), 4.06–4.02 (1H, m, H_b-(C3)), 2.06 (3H, s, C<u>H</u>$_3$). ^{13}C NMR (176 MHz, H_2O) δ 174.1 (<u>C</u>O), 117.7 (C1), 62.8 (d, *J* = 4.5 Hz, C3), 42.4 (d, *J* = 7.3 Hz, C2), 21.8 (d, *J* = 16.2 Hz, CH_3). ^{31}P NMR (284 MHz, H_2O, pH 4, ^{1}H decoupled) δ 1.76. HRMS-ESI $[M+H^+]^+$ calculated for formula $C_5H_{10}N_2O_5P^+$, 209.0322; found 209.0327.

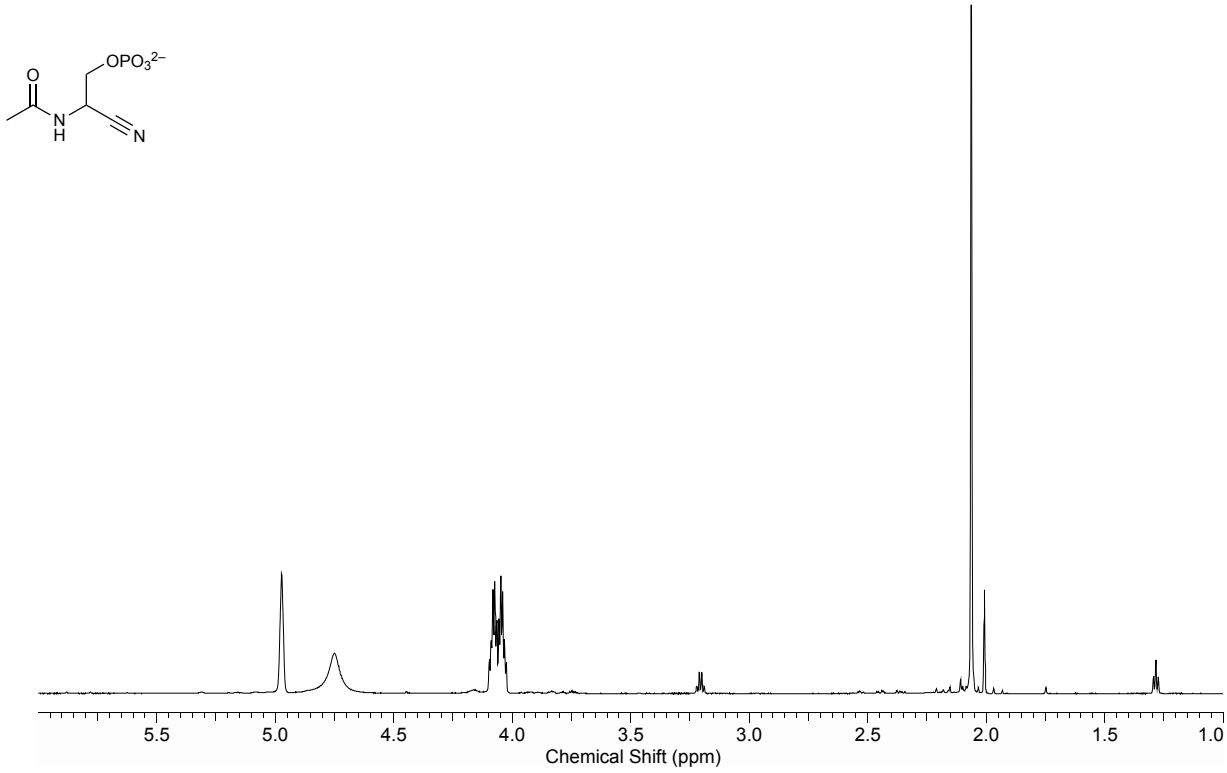


Fig. S26. ^{1}H NMR (700 MHz, H_2O, noesygppr1d, 1.00 – 6.00 ppm) spectrum to show **Ac-Sep-CN**.

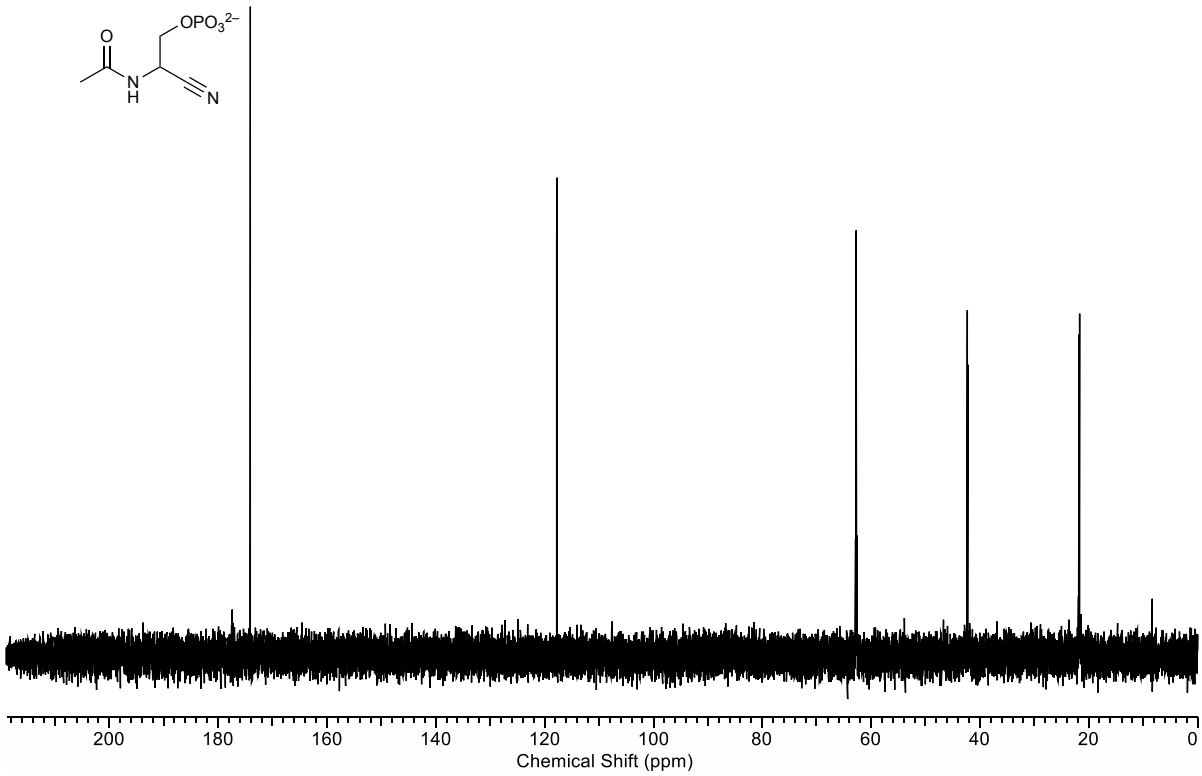
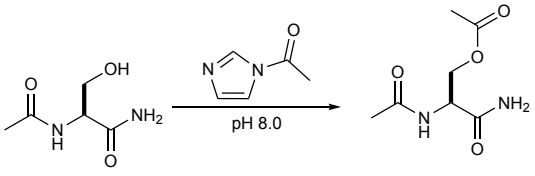


Fig. S27. ^{13}C NMR (176 MHz, H_2O, 0 – 220 ppm) spectrum to show **Ac-Sep-CN**.

Synthesis of N,O-diacetylserinamide



Ac-Ser-NH$_2$ **Ac-Ser^{Ac}-NH$_2$**

N-Acetyl-L-serinamide **Ac-Ser-NH$_2$** (146 mg, 1.00 mmol) and *N*-acetyl imidazole (450 mg, 4.08 mmol) were dissolved in water (10 mL) and the solution was adjusted to pH 8 with 4 M HCl. The solution was maintained at pH 8 until all *N*-acetylimidazole had dissolved. The reaction was incubated for 4 h at room temperature and then lyophilised. The lyophilite was then purified by flash column chromatography (SiO_2; eluting with petroleum ether/ethyl acetate 9:1 to 0:1, followed by ethyl acetate/methanol 100:0 to 1:1) to yield *N,O*-diacetyl-L-serinamide **Ac-Ser^{Ac}-NH$_2$** as a white solid which was contaminated with imidazole. The crude solid was dissolved in water and H^+-Dowex® was added until the solution was observed to be at pH 3.2. The solution was filtered then and concentrated *in vacuo* to give **Ac-Ser^{Ac}-NH$_2$** as a white solid (141 mg) that was contaminated with residual **Ac-Ser-NH$_2$** (3%) and acetate ($^-CO_2CH_3$, 58%). The compound was used without further purification. ^{1}H NMR (700 MHz, D_2O) δ 4.62 (1H, dd, *J* = 5.4, 4.1 Hz, H-(C2)), 4.44 (1H, ABX, *J* = 11.6, 5.4 Hz, H_a-(C3)), 4.32 (1H, ABX, *J* = 11.6, 4.1 Hz, H_b-(C3)), 2.09 (3H, s, **C**H_3CO_2), 2.05 (3H, s, **C**H_3CONH). ^{13}C NMR (176 MHz, H_2O) δ 174.5 (CH_3**C**ONH), 173.8 (C1), 173.6 (CH_3**C**O_2), 63.6 (C3), 52.6 (C2), 21.8 (**C**H_3CO_2), 20.1 (**C**H_3CONH). IR (cm^{-1}): 3165, 3061, 1744, 1671, 1657, 1559. HRMS-ESI $[M+H^+]^+$ calculated for formula $C_7H_{13}N_2O_4^+$, 189.0867; found 189.0875.

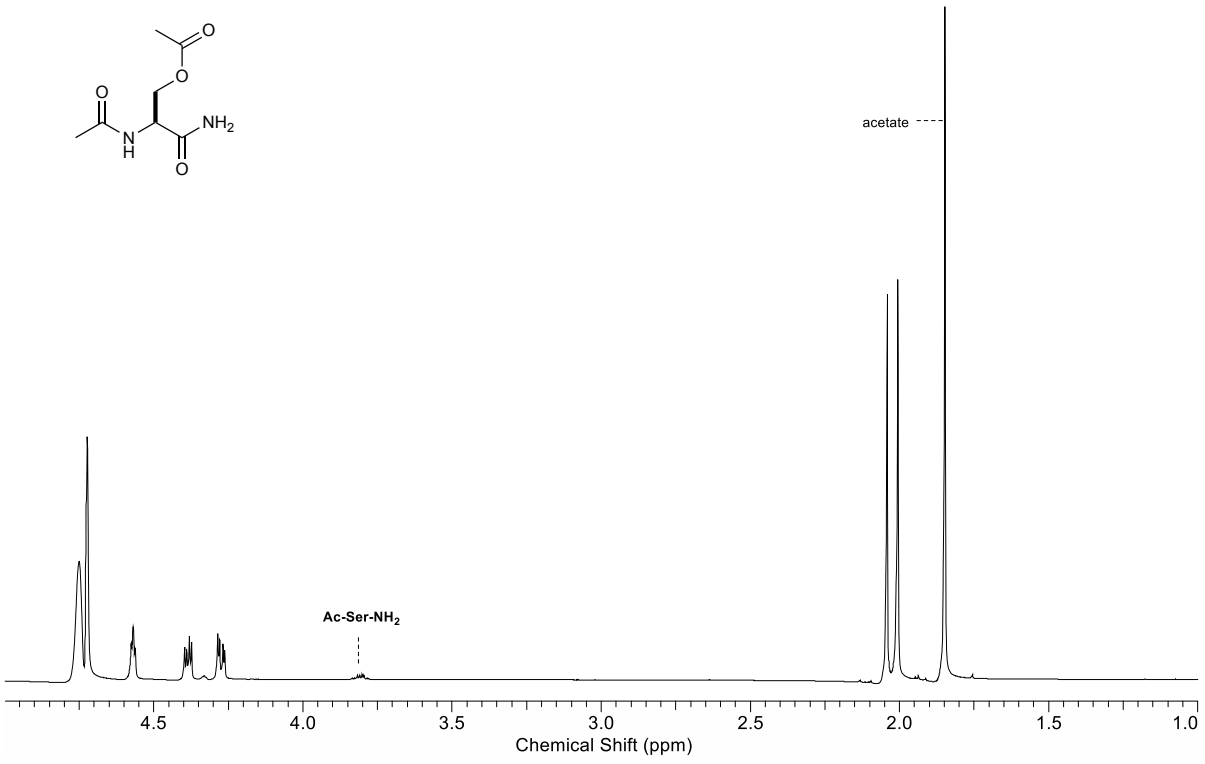


Fig. S28. ^{1}H NMR (700 MHz, H_2O, noesygppr1d, 1.00 – 5.00 ppm) spectrum to show **Ac-Ser^{Ac}-CN**.

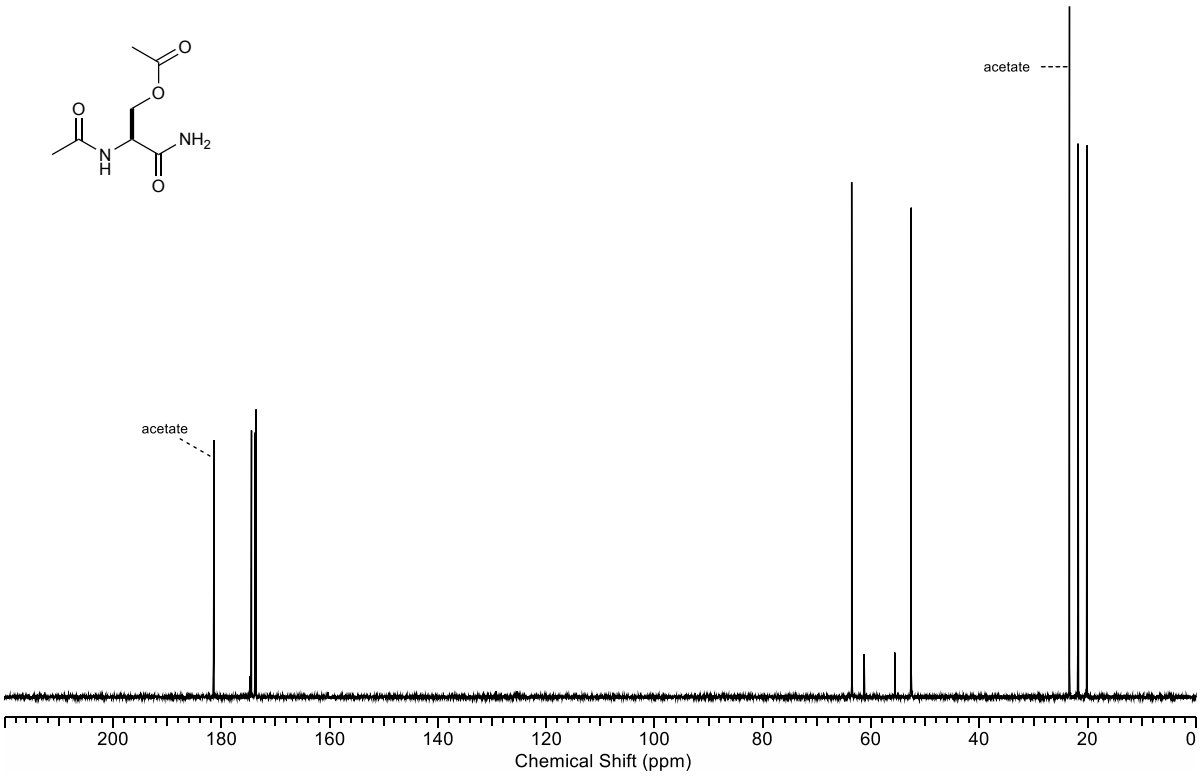
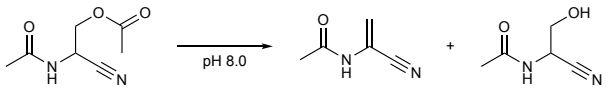


Fig. S29. ^{13}C NMR (176 MHz, H_2O, 0 – 220 ppm) spectrum to show **Ac-Ser^{Ac}-CN**.

Synthesis of N-acetyldehydroalanine nitrile from N,O-diacetyl-DL-serine nitrile in water



Ac-Ser^{Ac}-CN **Ac-Dha-CN** **Ac-Ser-CN**

N,O-Diacetyl-DL-serine nitrile **Ac-Ser^{Ac}-CN** (500 mg, 2.94 mmol) was dissolved in water at pH 8. The solution pH was monitored and periodically readjusted to pH 8 with NaOH. After 4 d the reaction composition was **Ac-Dha-CN**:**Ac-Ser^{Ac}-CN**:**Ac-Ser-CN** 85:10:5. The reaction was lyophilised and the residue was purified by flash column chromatography (SiO_2; eluting with a gradient of petroleum ether/ethyl acetate 9:1 to 1:9*). N*-Acetyldehydroalanine nitrile **Ac-Dha-CN** was obtained as a colourless solid (213 mg, 66%). ^{1}H NMR (700 MHz, D_2O) δ 5.84 (1H, s, H_a-(C3)), 5.73 (1H, s, H_b-(C3)), 2.05 (1H, s, 3H, $COCH_3$). ^{13}C NMR (176 MHz, D_2O) δ 173.4 ($\underline{C}OCH_3$), 121.5 (C3), 116.0 (C2), 115.6 (C1), 22.7 ($CO\underline{C}H_3$). IR (cm^{-1}) 2925, 2359, 2349, 1599. HRMS-ESI $[M+H^+]^+$ calculated for formula $C_5H_7N_2O^+$, 111.0553; found 111.0548.

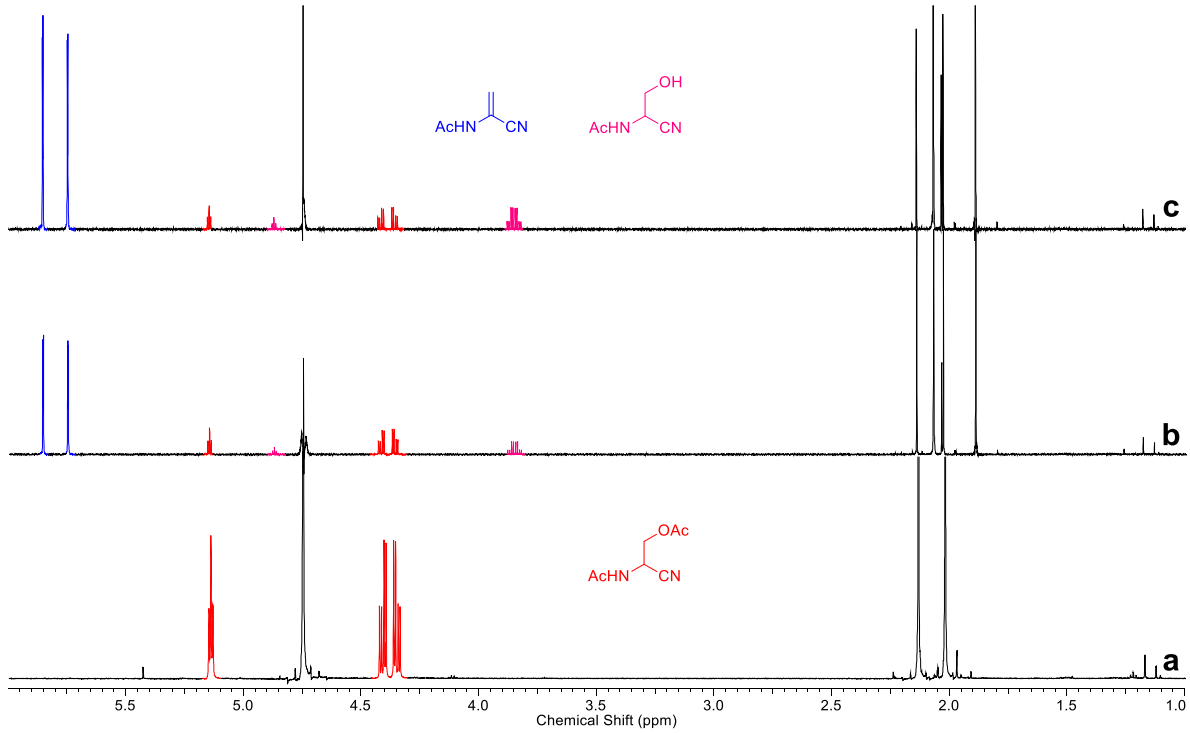


Fig. S30. ^{1}H NMR spectra (700 MHz, H_2O, noesygppr1d, 1.00–6.00 ppm) to show: a) *N,O*-diacetyl-DL-serine nitrile **Ac-Ser^{Ac}-CN** (100 mM) at pH 7; b) incubation of **Ac-Ser^{Ac}-CN** (100 mM) at pH 8 for 3 d; c) incubation of **Ac-Ser^{Ac}-CN** (100 mM) at pH 8 for 4 d.

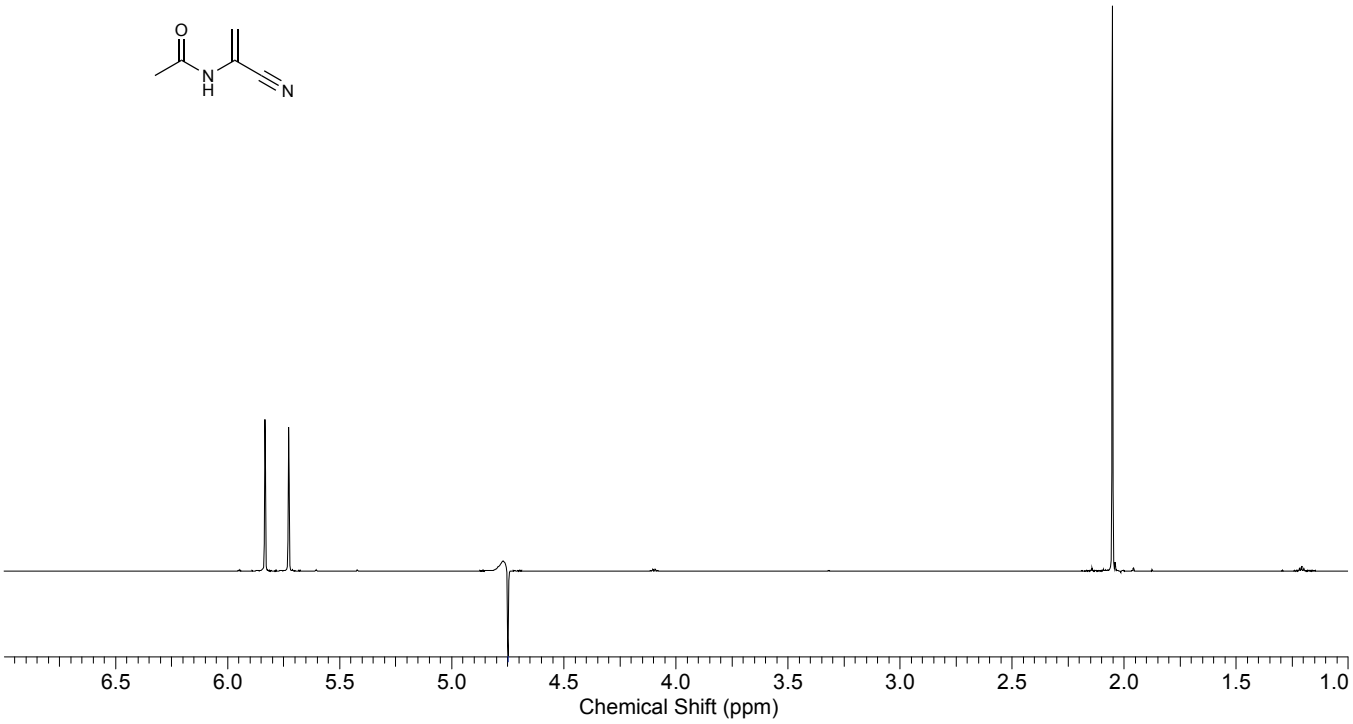


Fig. S31. ^{1}H NMR (700 MHz, D_2O, 1.0–7.0 ppm) spectrum to show *N*-acetyldehydroalanine nitrile **Ac-Dha-CN**.

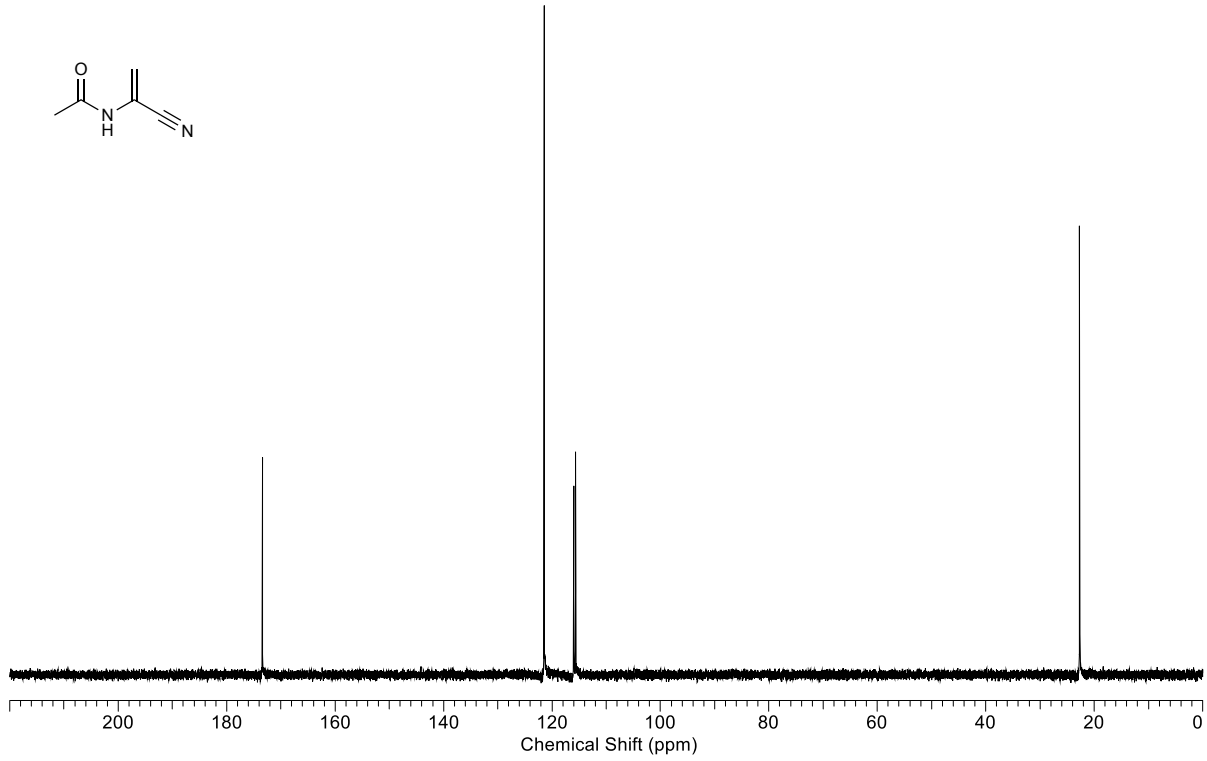
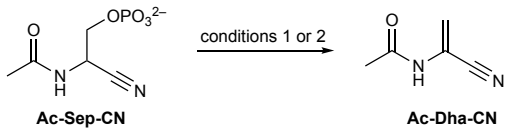


Fig. S32. ^{13}C NMR (176 MHz, D_2O, 0–220 ppm) spectrum to show *N*-acetyldehydroalanine nitrile **Ac-Dha-CN**.

Synthesis of N-acetyldehydroalanine nitrile from N-acetyl-O-phospho-DL-serine nitrile in water



1. phosphate buffer (500 mM), pH 7, 60 °C
2. $MgCl_2$ (250 mM), pH 7, 60 °C

N-Acetyl-*O*-phospho-DL-serine nitrile **Ac-Sep-CN** (50 mM) was dissolved in phosphate buffer (500 mM) (condition 1) or magnesium chloride solution ($MgCl_2$; 250 mM) at pH 7 (condition 2). The resulting solutions were heated at 60 °C and monitored periodically by NMR spectroscopy. The formation of **Ac-Dha-CN** (8%) was observed in phosphate buffer after 3 d (Fig. S33b). The formation of **Ac-Dha-CN** (24%) was also observed in the presence of $MgCl_2$ (Fig. S33c) after 24h.

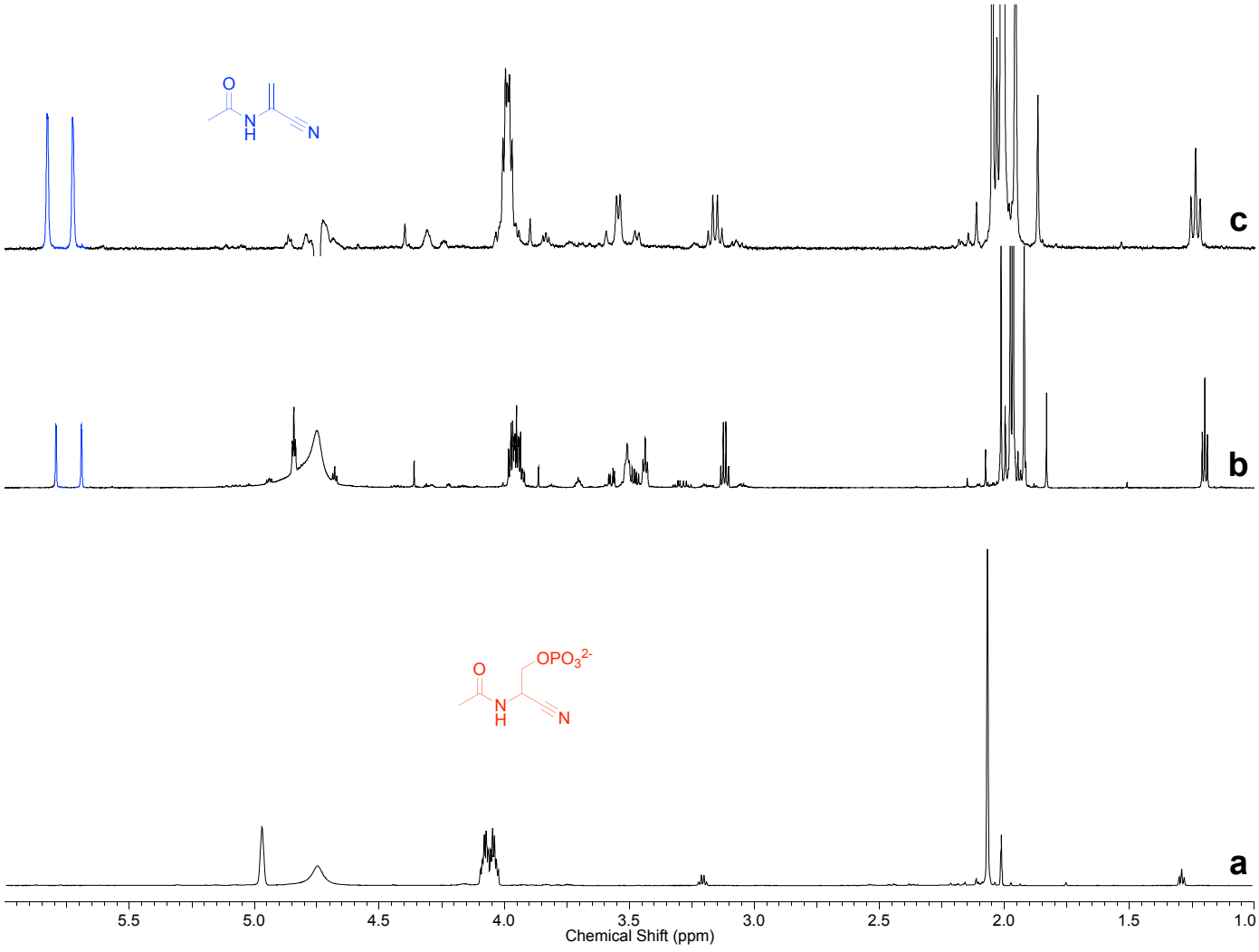
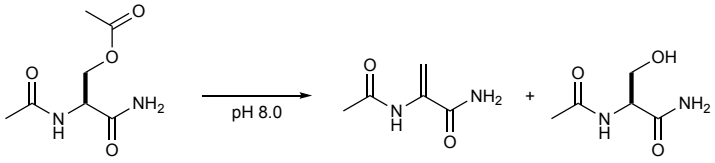


Fig. S33. ^1H NMR spectra (700 MHz, H_2O, noesygppr1d, 1.00–6.00 ppm) to show: **a**) **Ac-Sep-CN** (50 mM) in phosphate buffer at pH 7 and room temperature; **b**) incubation of **Ac-Sep-CN** (50 mM) in phosphate buffer (500 mM) at pH 7 and 60 °C for 3 d; **c**) incubation of **Ac-Sep-CN** (50 mM) in magnesium chloride solution ($MgCl_2$; 250 mM) at pH 7 and 60 °C for 24 h.

Attempted N-acetyldehydroalaninamide formation from N,O-diacetyl-L-serinamide



N,*O*-Diacetyl-L-serinamide **Ac-Ser^{Ac}-NH$_2$** (100 mM) was dissolved in water at pH 8. The reaction was monitored by periodic NMR spectroscopy. The solution pH was monitored and maintained at pH 8. Ester hydrolysis of **Ac-Ser^{Ac}-NH$_2$** to *N*-acetyl-L-serinamide **Ac-Ser-NH$_2$** (35%) predominated after 6 h, with only trace amounts of *N*-acetyl-L-dehydroalaninamide **Ac-Dha-NH$_2$** (<1%) present (Fig. S34), as observed by Jencks and co-workers (*32*). A minor species was observed but not characterised (•) (3%). This was also observed during the incubation of *N*-acetyl-DL-serine nitrile **Ac-Ser^{Ac}-CN** and **Ac-Ser^{Ac}-NH$_2$** at pH 8.0 (Fig. S35).

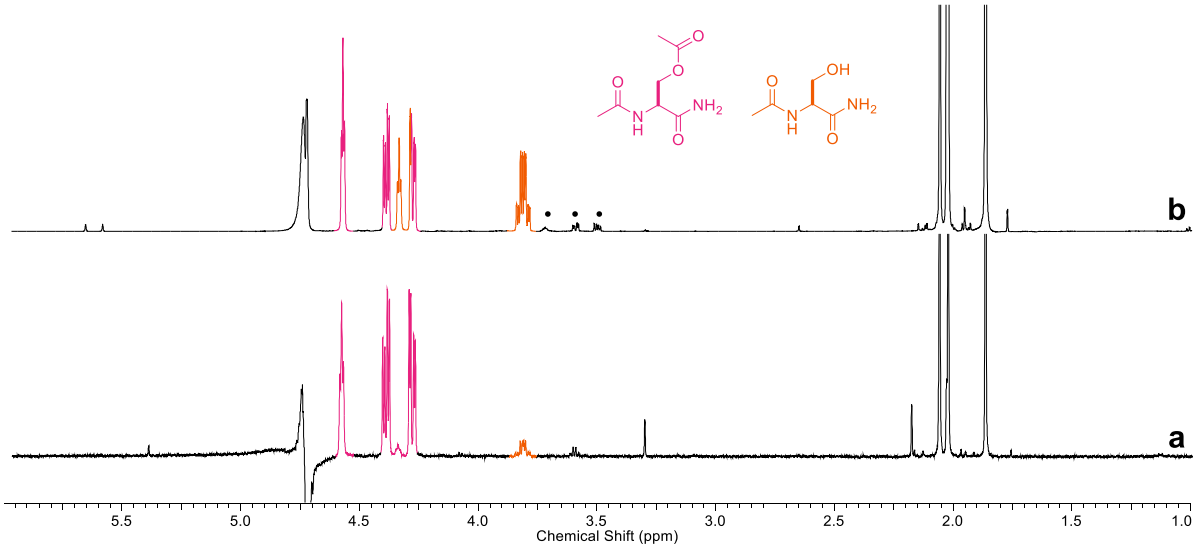
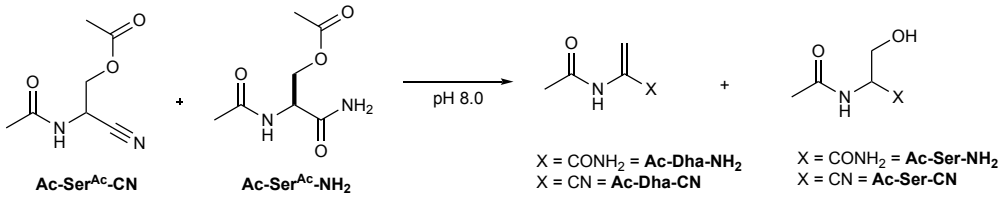


Fig. S34. ^{1}H NMR spectra (700 MHz, H_2O, noesygppr1d, 1.00–6.00 ppm) to show: **a**) **Ac-Ser^{Ac}-NH$_2$** (100 mM) at pH 7; **b**) incubation of **Ac-Ser^{Ac}-NH$_2$** (100 mM) at pH 8 for 6 h.

N-Acetyldehydroalanine nitrile formation from N,O-diacetyl-DL-serine nitrile in the presence of N,O-diacetyl-L-serinamide



Ac-Ser^{Ac}-CN **Ac-Ser^{Ac}-NH$_2$**

N,*O*-Diacetyl-DL-serine nitrile **Ac-Ser^{Ac}-CN** (100 mM) and *N*,*O*-diacetyl-L-serinamide **Ac-Ser^{Ac}-NH$_2$** (100 mM) were dissolved in water and the solution was adjusted to pH 8 with HCl/NaOH. The reaction was monitored periodically by NMR spectroscopy and the solution maintained at pH 8 with periodic addition of 0.1 M NaOH. After 24 h, the conversion of **Ac-Ser^{Ac}-CN** to **Ac-Dha-CN** (77%) was observed. **Ac-Ser^{Ac}-NH$_2$** underwent ester hydrolysis to give **Ac-Ser-NH$_2$** (25%) (Fig. S35), as observed by Jencks and co-workers (*32*). A minor species was observed but not characterised (•) (19%). This was also observed during the incubation of **Ac-Ser^{Ac}-NH$_2$** only at pH 8.0 (see Fig. S34).

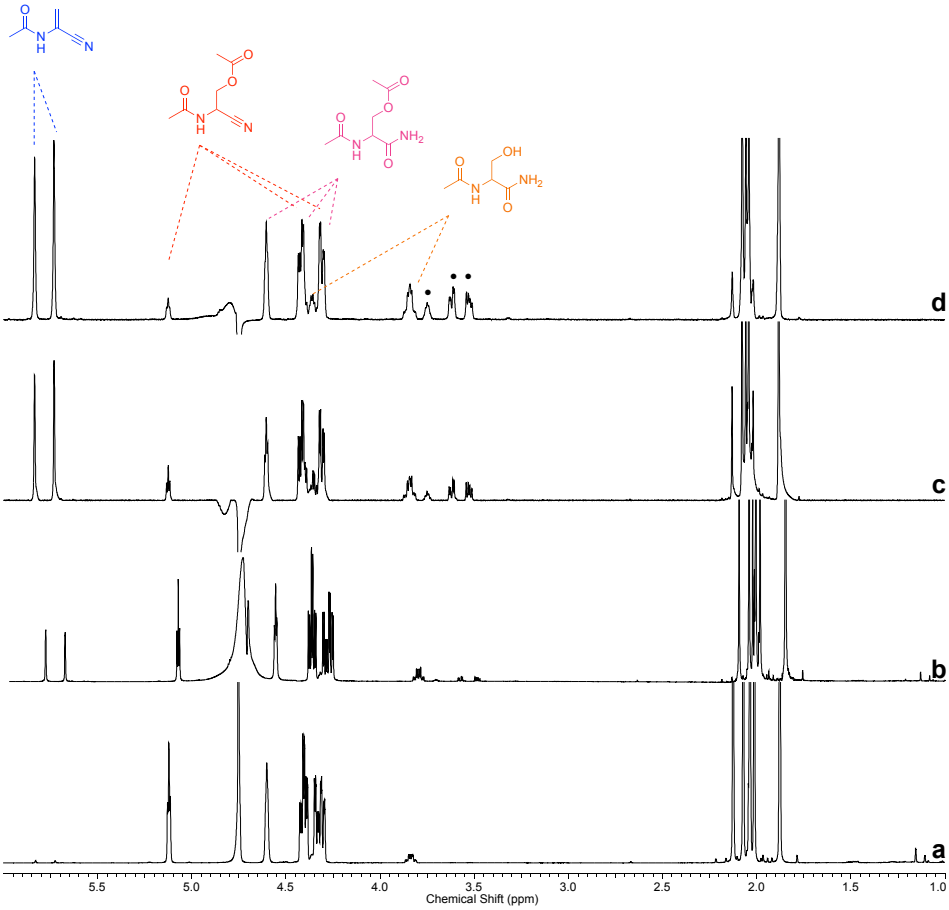
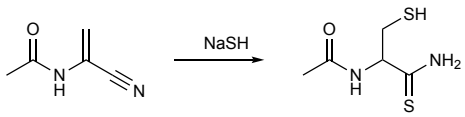


Fig. S35. ^{1}H NMR spectra (600 MHz, H_2O, noesygppr1d, 1.00–6.00 ppm) to show: **a**) **Ac-Ser^{Ac}-CN** (100 mM) and **Ac-Ser^{Ac}-NH$_2$** (100 mM) at pH 7; **b**) incubation of **Ac-Ser^{Ac}-CN** (100 mM) and **Ac-Ser^{Ac}-NH$_2$** (100 mM) at pH 8 after 8 h; **c**) incubation of **Ac-Ser^{Ac}-CN** (100 mM) and **Ac-Ser^{Ac}-NH$_2$** (100 mM) at pH 8 after 16 h; **d**) incubation of **Ac-Ser^{Ac}-CN** (100 mM) and **Ac-Ser^{Ac}-NH$_2$** (100 mM) at pH 8 after 24 h.

Addition of hydrogen sulfide to N-acetydehydroalanine nitrile



N-Acetyldehydroalanine nitrile **Ac-Dha-CN** (60 mM) was dissolved in water and the solution sparged with argon for 30 min. Sodium hydrosulfide (NaSH·×H_2O; 10 equiv.) was then added, the solution was adjusted to the specified pH with HCl/NaOH, and the reactions were incubated at room temperature. The reaction mixtures were analysed by NMR spectroscopy after 4 h. The formation of *N*-acetyl-DL-cysteine thioamide **Ac-Cys-SNH_2** (>95%) was observed (Fig. S36). ^{1}H NMR 700 MHz, D_2O) δ 4.42 (dd, *J* = 7.9, 4.7 Hz, 1H, H-(C2)), 3.00 (ABX, *J* = 13.2, 4.7 Hz, 1H, H_a-(C3)), 2.84 (ABX, *J* = 13.2, 7.9 Hz, 1H, H_b-(C3)), 2.09 (s, 3 H, CH_3). ^{13}C NMR (176 MHz, H_2O) δ 205.3 (C1), 174.1 (CO), 64.6 (C2), 29.7 (C3), 22.0 (CH_3).

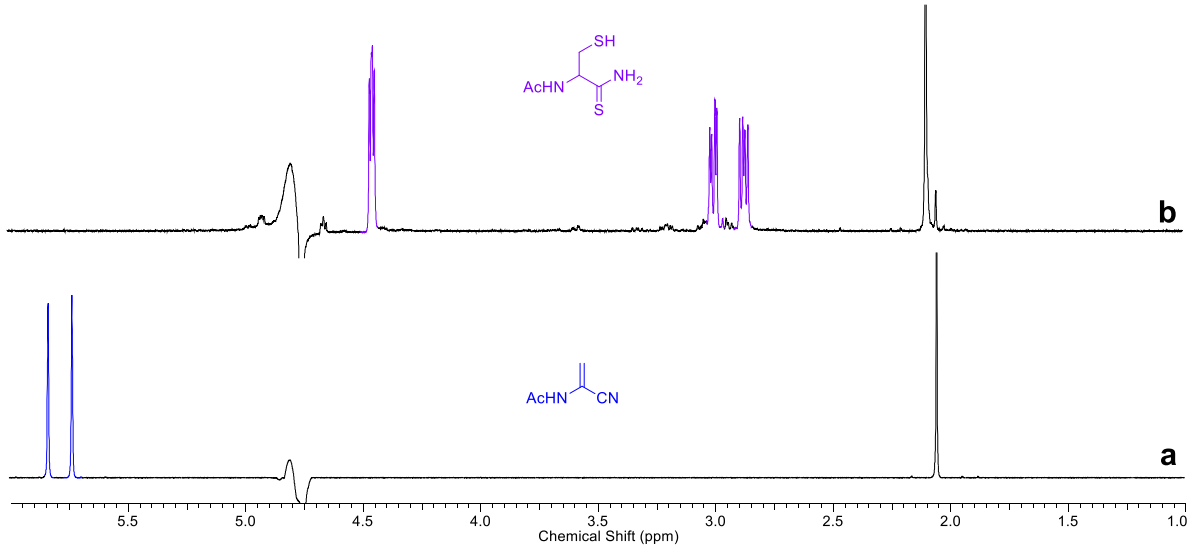


Fig. S36. ^{1}H NMR spectra (700 MHz, H_2O, noesygppr1d, 1.00–6.00 ppm) to show: **a**) **Ac-Dha-CN** (60 mM) at pH 7.0; **b)** the reaction of **Ac-Dha-CN** (60 mM) with NaSH (10 equiv.) at room temperature and pH 9 after 4 h.

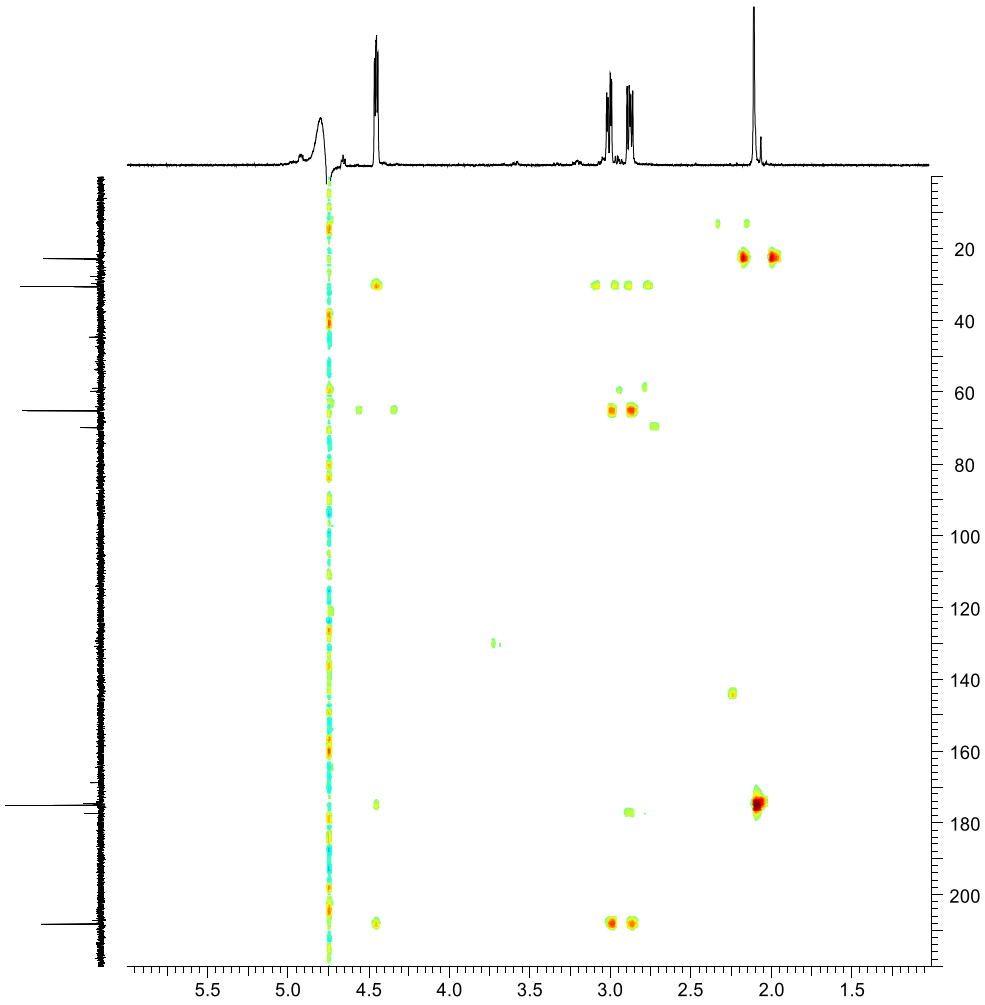
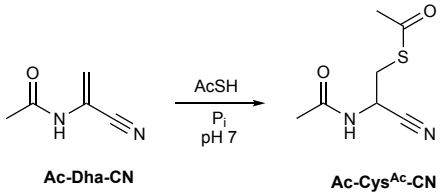


Fig. S37. ^{1}H–^{13}C HMBC (^{1}H: 700 MHz [1.00–6.00 ppm], ^{13}C: 176 MHz [0-220 ppm], H_2O) to show the reaction of **Ac-Dha-CN** (60 mM) with NaSH (10 equiv.) at room temperature and pH 9 after 4 h showing the diagnostic $^{1}J_{CH}$ and $^{2}J_{CH}$ coupling of Cys-(C3)–H_2 of **Ac-Cys-SNH$_2$** at 2.78–3.07 ppm with the Cys-(C3) and Cys-(C2) resonances at 29.7 ppm and 64.6 ppm, respectively.

Addition of thioacetic acid to N-acetyldehydroalanine nitrile



Ac-Dha-CN (100 mM) and thioacetic acid (**AcSH**, 4 equiv.) were incubated in phosphate buffer (500 mM, pH 7). The resulting solution was periodically analysed by NMR spectroscopy. Quantitative (>95%) conversion to *N*,*S*-diacetyl-DL-cysteine nitrile **Ac-Cys^{Ac}-CN** was observed after 12 h (Fig. S38).

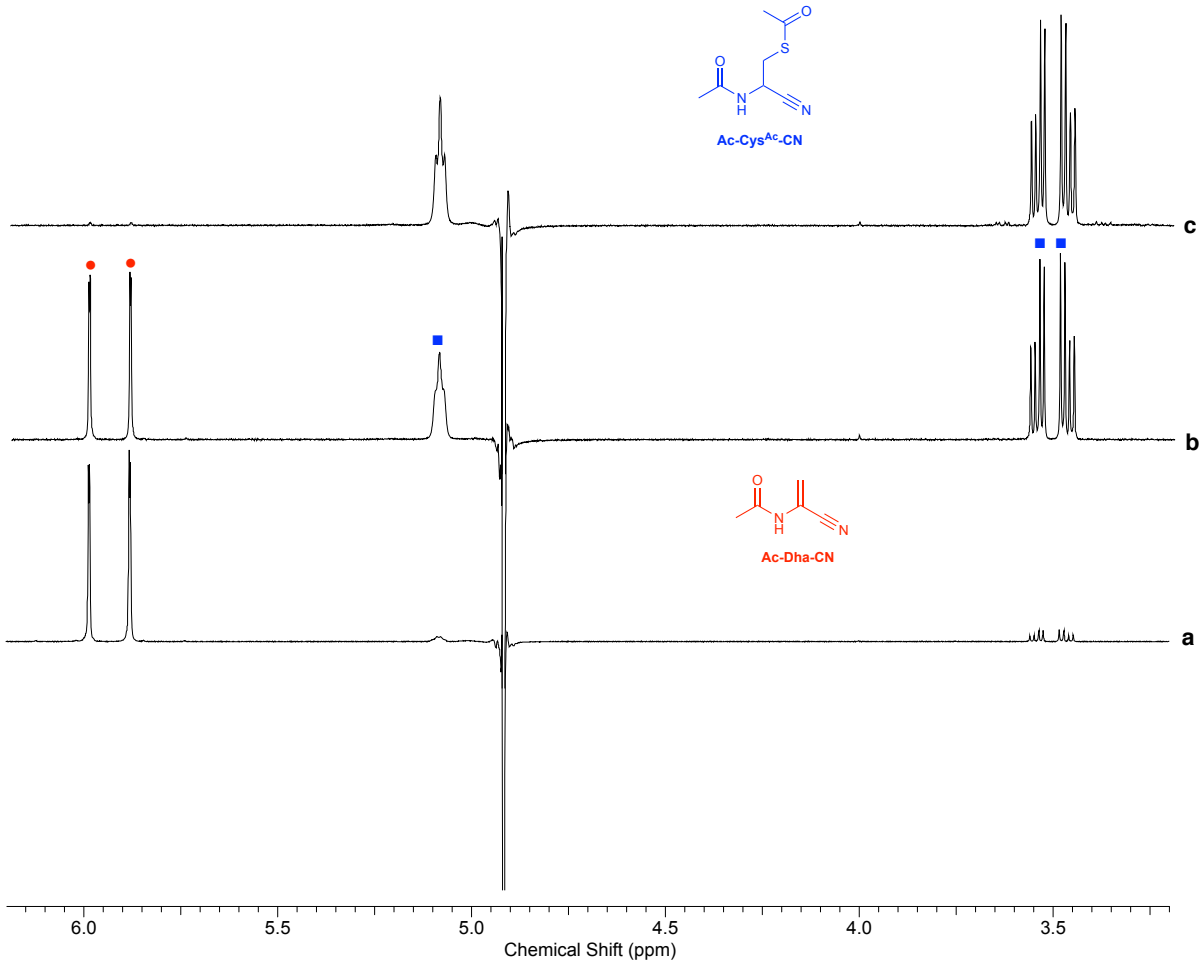
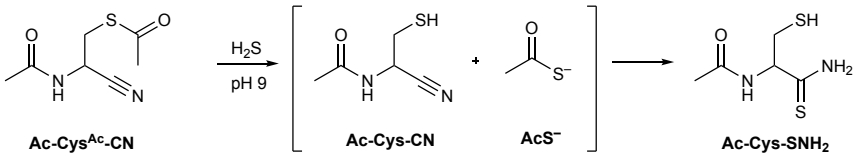


Fig. S38. Stacked ^{1}H NMR (600 MHz; H_2O, noesygppr1d) spectra to show the formation of *N,S*-diacetyl-DL-cysteine nitrile (**Ac-Cys^{Ac}-CN**) by reaction of *N*-acetyldehydroalanine nitrile (**Ac-Dha-CN**; 100 mM) with thioacetic acid (**AcSH**; 400 mM) in phosphate buffer (500 mM) at pH 7 and room temperature. **a** = 10 min; **b** = 2 h; **c** = 12 h.

Addition of hydrogen sulfide to N,S-diacetyl-DL-cysteine nitrile



N,*S*-Diacetyl-DL-cysteine nitrile **Ac-Cys^{Ac}-CN** (50 mM) was dissolved in water and the solution was sparged with argon for 30 min. The solution was adjusted to pH 9.0 with HCl/NaOH. Sodium hydrosulfide (NaSH·×H_2O; 10 equiv.) was then added at pH 9 at room temperature, and the reaction was monitored periodically by NMR spectroscopy. The thiolysis of **Ac-Cys^{Ac}-CN** to **Ac-Cys-CN** (58%) and thioacetate **AcS$^-$** was observed after 1.5 h, alongside *N*-acetyl-DL-cysteine thioamide **Ac-Cys-SNH$_2$**. Further incubation led to **Ac-Cys-SNH$_2$** (95%) after 4 h (Fig. S40).

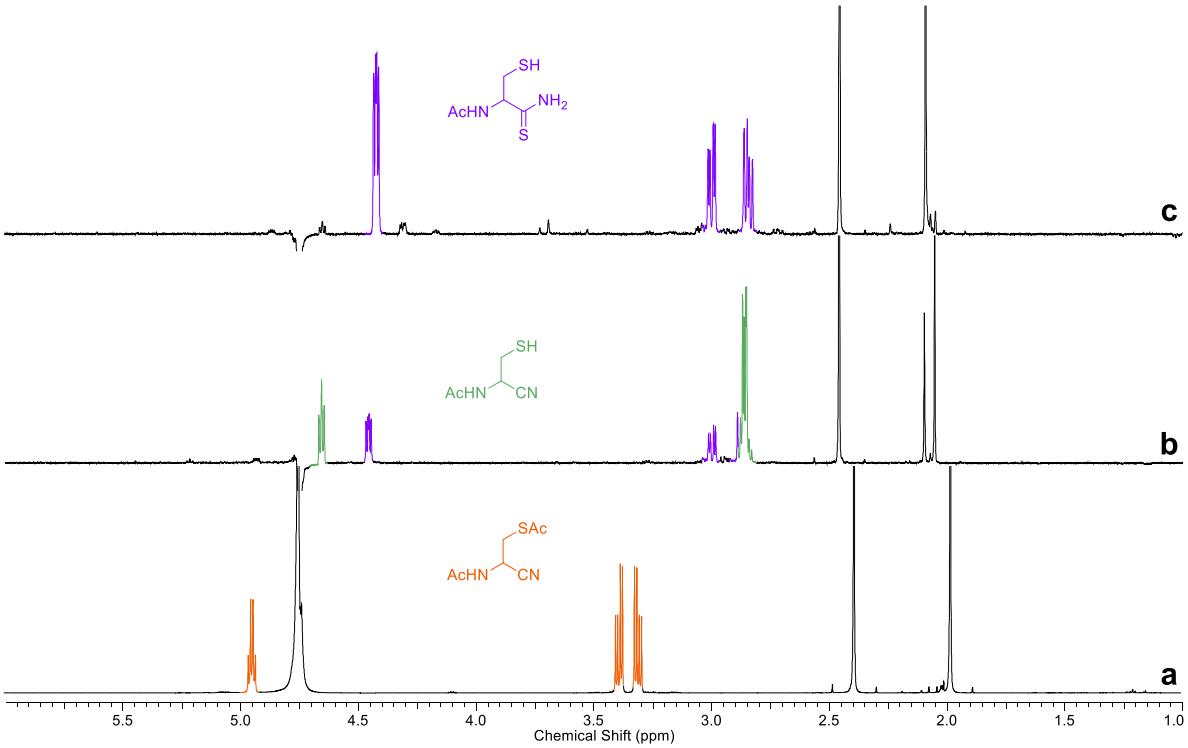
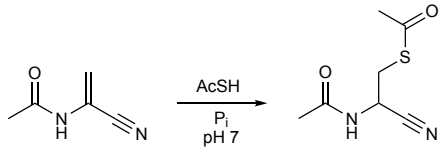


Fig. S39. ^{1}H NMR spectra (700 MHz, H_2O, noesygppr1d, 1.00–6.00 ppm) to show: **a**) **Ac-Cys^{Ac}-CN** (50 mM); **b**) the reaction of **Ac-Cys^{Ac}-CN** (50 mM) with NaSH (10 equiv.) at pH 9 and room temperature after 1.5 h; **c**) the reaction of **Ac-Cys^{Ac}-CN** (50 mM) with NaSH (10 equiv.) at pH 9 and room temperature after 4 h.

Preparative synthesis and isolation of N,S-diacetyl-DL-cysteine nitrile



Ac-Dha-CN **Ac-Cys^{Ac}-CN**

N-Acetyldehydroalanine nitrile **Ac-Dha-CN** (74 mg, 0.67 mmol) was dissolved in 500 mM phosphate buffer (pH 7, 6.7 mL) and thioacetic acid (189 μL, 0.27 mmol) was added. The reaction was incubated at room temperature and monitored by ^{1}H NMR spectroscopy until quantitative conversion of **Ac-Dha-CN** to *N,S*-diacetyl-DL-cysteine nitrile **Ac-Cys^{Ac}-CN** was observed (12 h). The solution was then extracted with ethyl acetate (4 × 10 mL). The combined organics were washed with water (10 mL), dried over anhydrous sodium sulfate, filtered, and concentrated *in vacuo* to give *N,S*-diacetyl-DL-cysteine nitrile **Ac-Cys^{Ac}-CN** (124 mg, quantitative) as a light brown oil, which became a waxy solid upon standing at 4 °C. ^{1}H NMR (700 MHz, H_2O/D_2O 9:1) δ 4.95 (1H, app.q, *J* = 7.2 Hz, (C2)–H), 3.40 (1H, ABX, *J* = 14.2, 6.7 Hz, (C3)–H_a), 3.31 (1H, ABX, *J* = 14.2, 7.2 Hz, (C3)–H_b), 2.39 (3H, s, $SCOCH_3$), 1.98 (3H, s, $NCOCH_3$). ^{13}C NMR (176 MHz, H_2O/D_2O 9:1) δ 199.5 (**C**OS), 174.5 (**C**ONH), 118.3 (C1), 41.54 (C2), 30.7 (C3), 30.4 (SCO**C**H_3), 22.3 (NCO**C**H_3). IR (cm^{-1}) 3331, 3256, 2245, 1692, 1661, 1527. HRMS-ESI $[M+H^+]^+$ calculated for formula $C_7H_{11}N_2O_2S^+$, 187.0536; found 187.0531.

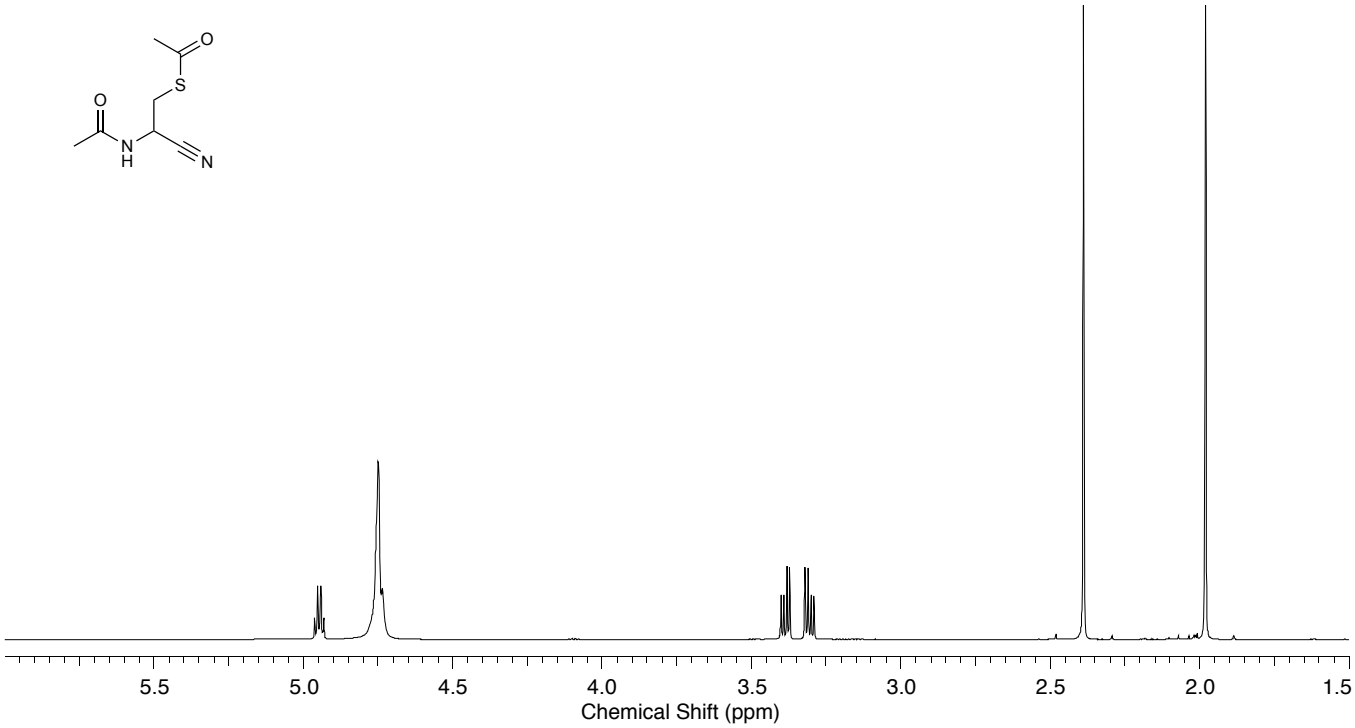


Fig. S40. ^{1}H NMR (700 MHz, H_2O/D_2O, 9:1, 1.5–6.0 ppm, noesygppr1d) spectrum to show *N,S*-diacetyl-DL-cysteine nitrile (**Ac-Cys^{Ac}-CN**).

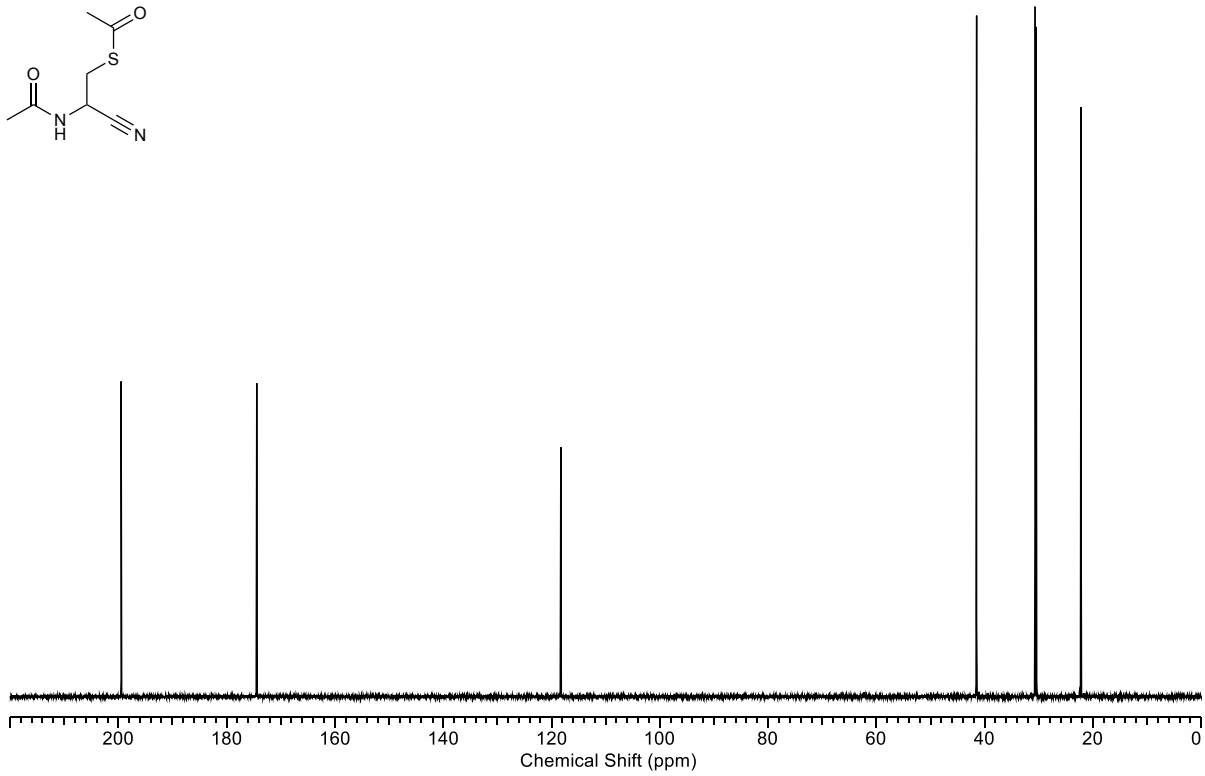
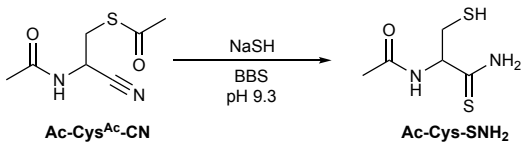


Fig. S41. ^{13}C NMR (176 MHz, H_2O/D_2O, 9:1, 0–220 ppm, noesygppr1d) spectrum to show *N,S*-diacetyl-DL-cysteine nitrile (**Ac-Cys^{Ac}-CN**).

Preparative synthesis and isolation of N-acetyl-DL-cysteine thioamide



Ac-Cys^{Ac}-CN **Ac-Cys-SNH$_2$**

N,S-Diacetyl-DL-cysteine nitrile **Ac-Cys^{Ac}-CN** (115 mg, 0.62 mmol) and sodium hydrosulfide hydrate (NaSH·×H$_2$O; 692 mg, 6.18 mmol) were dissolved in 1 M borate buffer (BBS; pH 9.3, 12.4 mL). The reaction was stirred at room temperature for 1 h. The formation of a white precipitate was observed (assumed to be a sodium borate salt). Tris(2-carboxyethyl)phosphine hydrochloride (**TCEP**·HCl; 177 mg, 1. equiv.) was added and the reaction stirred for 30 min. The solution was decanted from the white precipitate, and the filtrate was extracted with ethyl acetate (3 × 25 mL). The combined organic layers were washed with water (10 mL), dried over anhydrous sodium sulfate, filtered, and concentrated *in vacuo* to give *N*-acetyl-DL-cysteine thioamide **Ac-Cys-SNH$_2$** (81 mg) as an off-white gum. ^{1}H NMR (700 MHz, D$_2$O, noesygppr1d) δ 4.70 (ABX, *J* = 5.8, 6.7 Hz, 1H, (C2)–H), 3.01 (dd, *J* = 5.8, 14.1 Hz, 1H, (C3)–H$_a$), 2.98 (dd, *J* = 6.7, 14.1 Hz, 1H, (C3)–H$_b$), 2.05 (s, 3H, COCH$_3$). ^{13}C NMR (176 MHz, D$_2$O) δ 206.1 (C1), 175.0 (**C**OCH$_3$), 61.9 (C2), 28.2 (C3), 22.4 (CO**C**H$_3$). HRMS-ESI [M–H]$^-$ calculated for C$_5$H$_9$N$_2$OS$_2$ 177.0156; observed 177.0158.

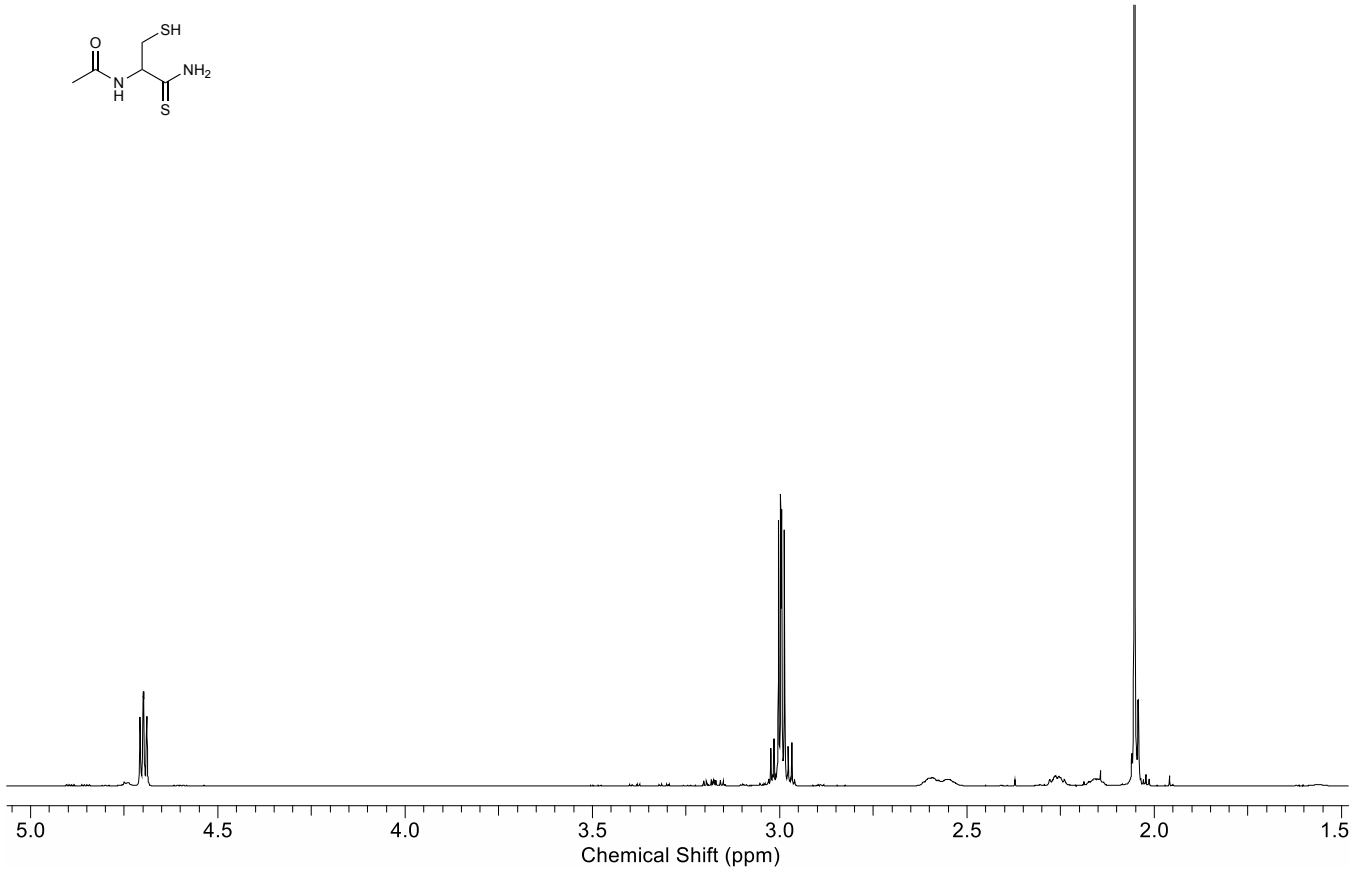


Fig. S42. ^{1}H NMR (700 MHz, D$_2$O, 1.5–5.0 ppm, noesygppr1d) spectrum to show *N*-acetyl-DL-cysteine thioamide (**Ac-Cys-SNH$_2$**).

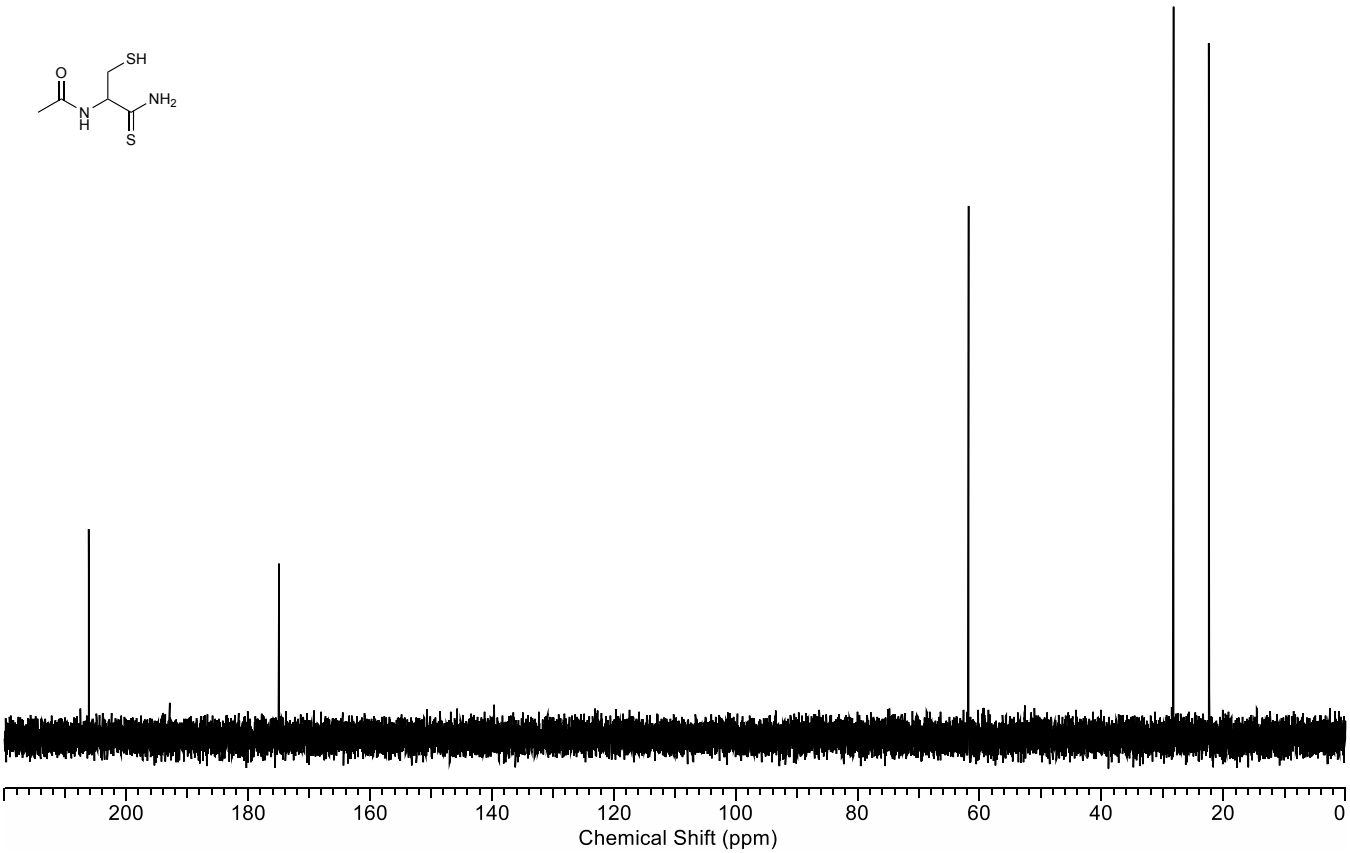
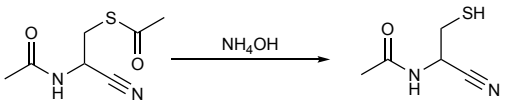


Fig. S43. ^{13}C NMR (176 MHz, D_2O, 0–220 ppm) spectrum to show *N*-acetyl-DL-cysteine thioamide (**Ac-Cys-SNH$_2$**).

Preparative synthesis and isolation of N-acetyl-DL-cysteine nitrile



Ac-Cys^{Ac}-CN **Ac-Cys-CN**

Ammonium hydroxide (25%; 275 μL) was added to *N*,*S*-diacetyl-DL-cysteine nitrile **Ac-Cys^{Ac}-CN** (111 mg; 0.60 mmol) in D_2O (10 mL). The reaction was incubated at room temperature and monitored by NMR spectroscopy. The conversion of **Ac-Cys^{Ac}-CN** to *N*-acetyl-DL-cysteine nitrile **Ac-Cys-CN** (77%) was observed within 10 min. The reaction solution was adjusted from pD 10.0 to pD 7.0 with 4M HCl and concentrated *in vacuo* (T_{bath}= 30 °C). The residue was extracted with dichloromethane (5 mL) and the organic extract was directly loaded onto a silica gel and purified by flash column chromatography (SiO_2; pet. ether (40-60):EtOAc; 25:75→0:100), to give *N*-acetyl-DL-cysteine nitrile **Ac-Cys-CN** (36 mg, 42%) as a colourless oil, which became a glassy solid upon storage at 4 °C. ^{1}H NMR (400 MHz, D_2O, noesygppr1d) δ 4.89 (app. q, *J* = 6.9 Hz, 1H, (C2)–H), 2.95 (d, *J* = 6.9 Hz, 1H, (C3)–H_2), 2.00 (s, 3H, $COCH_3$). ^{13}C NMR (176 MHz, D_2O δ 174.7 (**C**OCH_3), 118.7 (C1), 44.8 (C2), 26.4 (C3), 22.4 (CO**C**H_3). HRMS-ESI $[M+H]^+$ calculated for $C_5H_9N_2OS$ 145.0430; observed 145.0431. IR (cm^{-1}): 3242, 3051, 2245, 1648, 1636, 1540.

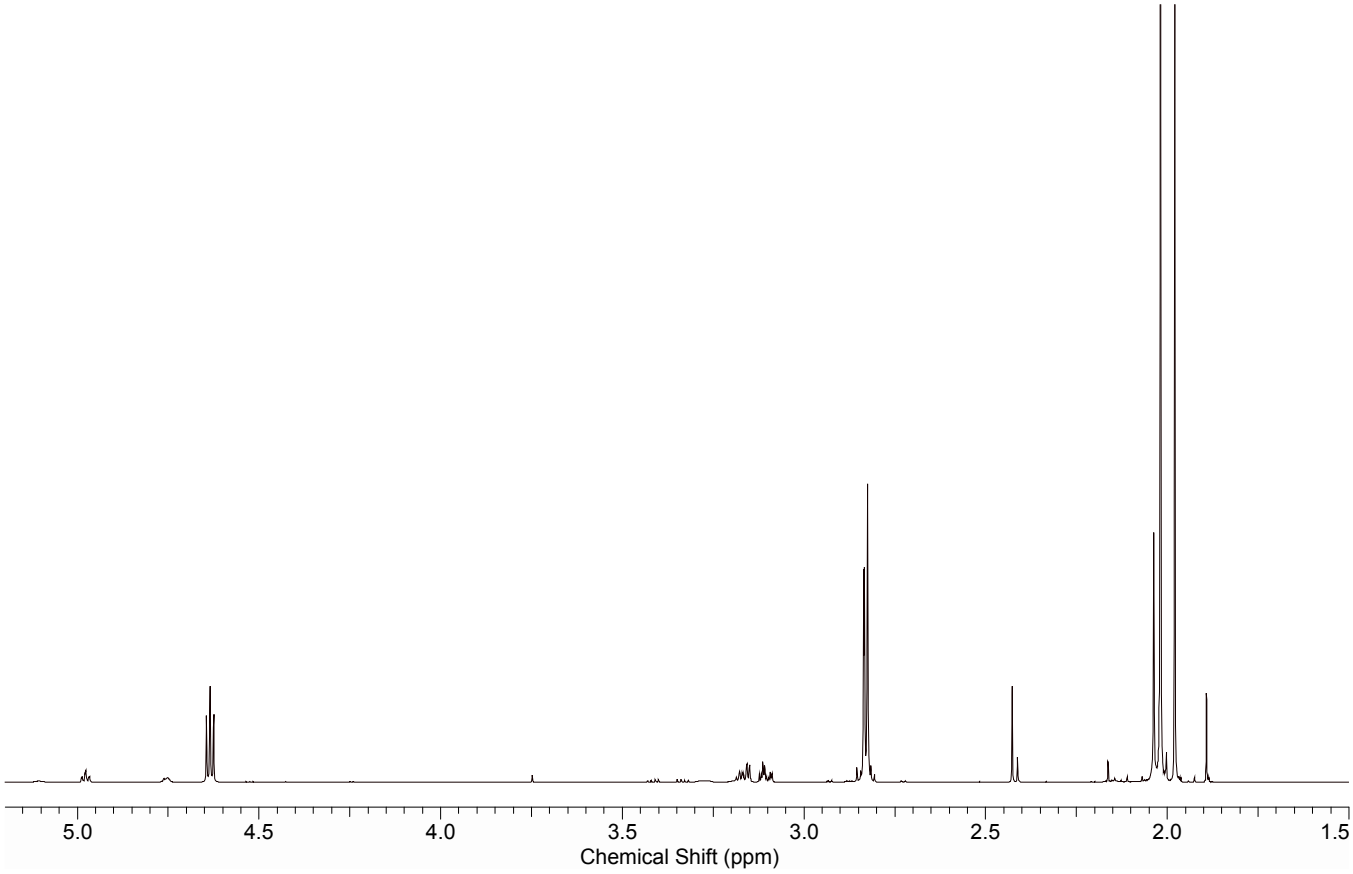


Fig. S44. ^{1}H NMR (700 MHz, D_2O, 1.5–5.2 ppm, noesygppr1d) spectrum to show the formation of *N*-acetyl-DL-cysteine nitrile(**Ac-Cys-CN**) after incubation of *N,S*-diacetyl-DL-cysteine nitrile (**Ac-Cys^{Ac}-CN**; 60 mM) with ammonia (360 mM) after 10 min at room temperature.

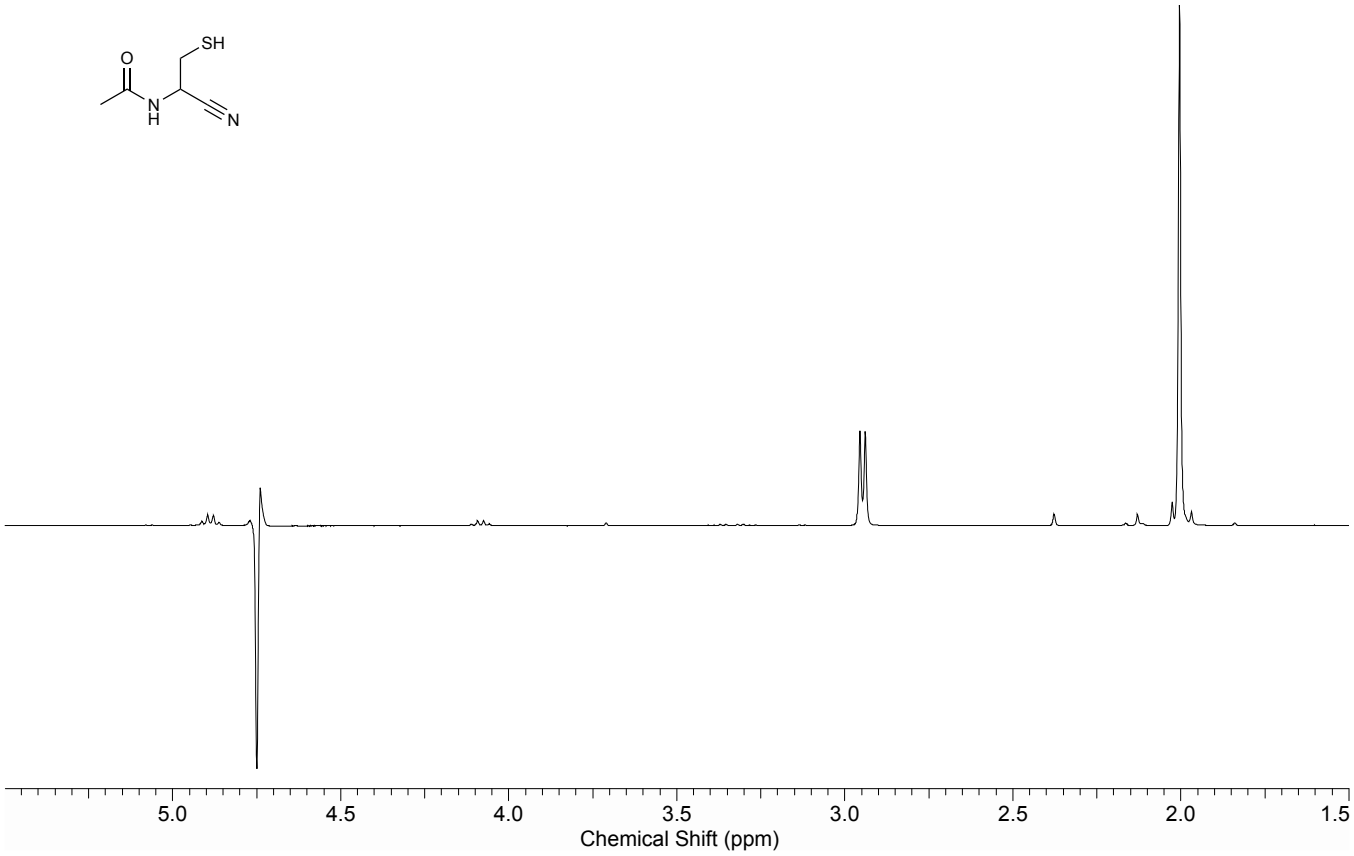


Fig. S45. ^{1}H NMR (400 MHz, D_2O, 1.5–5.5 ppm, noesygppr1d) spectrum to show *N*-acetyl-DL-cysteine nitrile(**Ac-Cys-CN**).

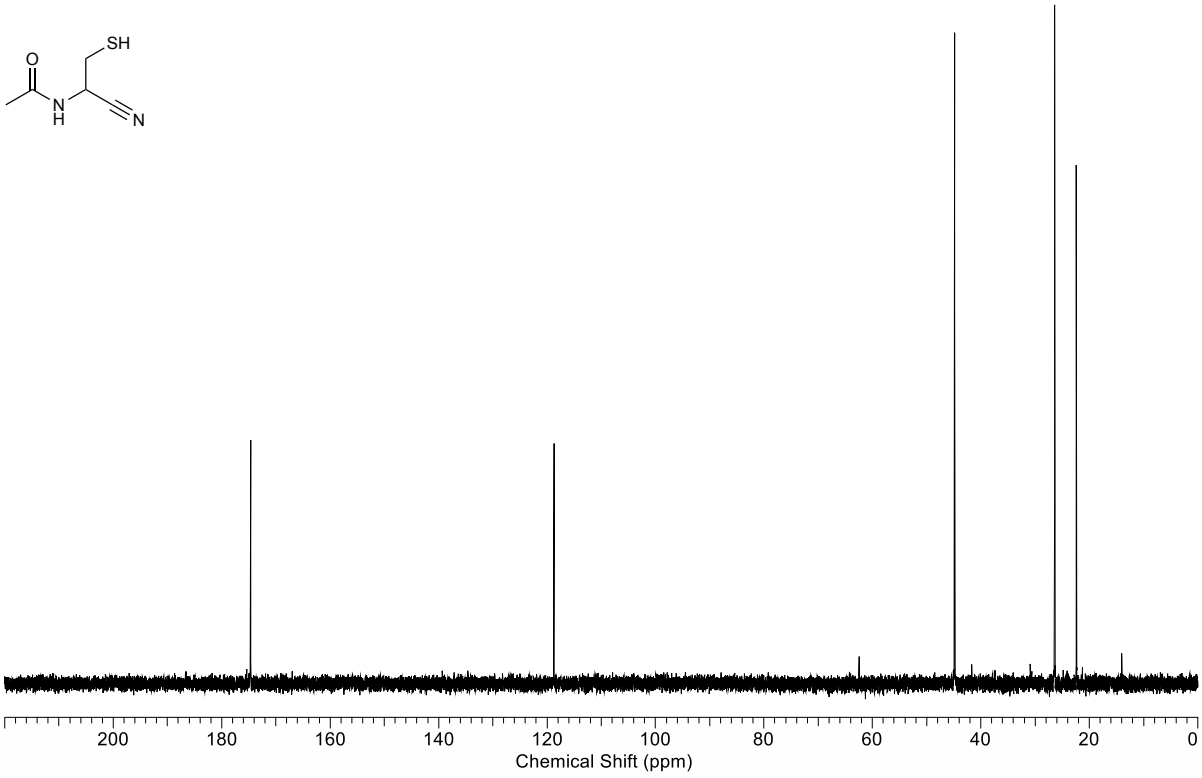
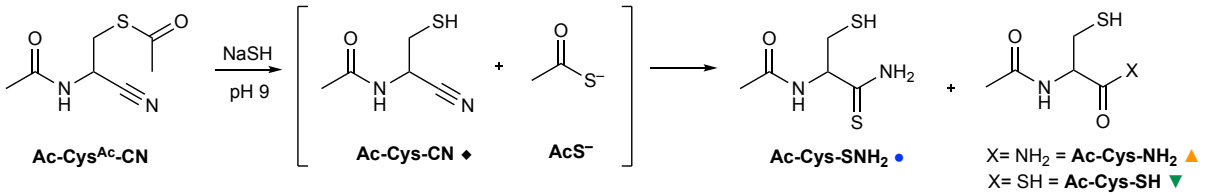


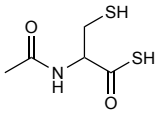
Fig. S46. ^{13}C NMR (176 MHz, D_2O, 0–220 ppm) spectrum to show *N*-acetyl-DL-cysteine nitrile (**Ac-Cys-CN**).

Incubation of N,S-diacetyl-DL-cysteine nitrile with hydrogen sulfide



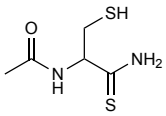
A solution of *N*,*S*-diacetyl-DL-cysteine nitrile **Ac-Cys^{Ac}-CN** (50 mM) and sodium hydrosulfide (NaSH•×H_2O; 500 mM) was adjusted to pH 9.0 with 4M HCl/NaOH. The reaction was incubated at room temperature and monitored by NMR spectroscopy. The conversion of **Ac-Cys^{Ac}-CN** to *N*-acetyl-DL-cysteine thioamide **Ac-Cys-SNH$_2$** (>95%) was observed after 16 h, followed by the gradual hydrolysis to a mixture of **Ac-Cys-SH** (25%) and **Ac-Cys-NH$_2$** (12%), with **Ac-Cys-SNH$_2$** (40%) remaining after 6 d (Fig. S47 and Fig. S48).

*Data for **Ac-Cys-SH***



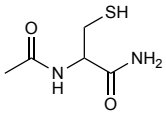
^{1}H NMR (700 MHz, H_2O) δ 4.32 (ddd, *J* = 1.1, 3.8, 9.5 Hz, 1H, Cys-(C2)–H), 3.05 (ddd, *J* = 1.3, 3.8, 13.1 Hz, 1H, Cys-(C3)–H), 2.72 (ddd, *J* = 1.3, 9.5, 13.1 Hz, 1H, Cys-(C3)–H′), 2.07 (d, *J* = 1.1 Hz, 3H, $COCH_3$).

*Data for N-acetyl-DL-cysteine thioamide **Ac-Cys-SNH$_2$***



^{1}H NMR (700 MHz, H_2O) δ 4.44 (ddd, *J* = 1.1, 4.9, 7.9 Hz, 1H, Cys-(C2)–H), 3.00 (ddd, *J* = 1.3, 4.9, 13.1 Hz, 1H, Cys-(C3)–H), 2.86 (ddd, *J* = 1.3, 7.9, 13.1 Hz, 1H, Cys-(C3)–H′), 2.10 (d, *J* = 1.1 Hz, 3H, $COCH_3$).

*Data for N-acetyl-DL-cysteineamide **Ac-Cys-NH$_2$***



^{1}H NMR (700 MHz, H_2O) δ 4.17 (ddd, *J* = 1.1, 5.1, 7.5 Hz, 1H, Cys-(C2)–H), 2.90 – 2.87 (obs., Cys-(C3)–H), 2.80 (ddd, *J* = 1.5, 7.5, 13.2 Hz, 1H, Cys-(C3)–H′), 2.09 (d, *J* = 1.5 Hz, 3H, $COCH_3$).

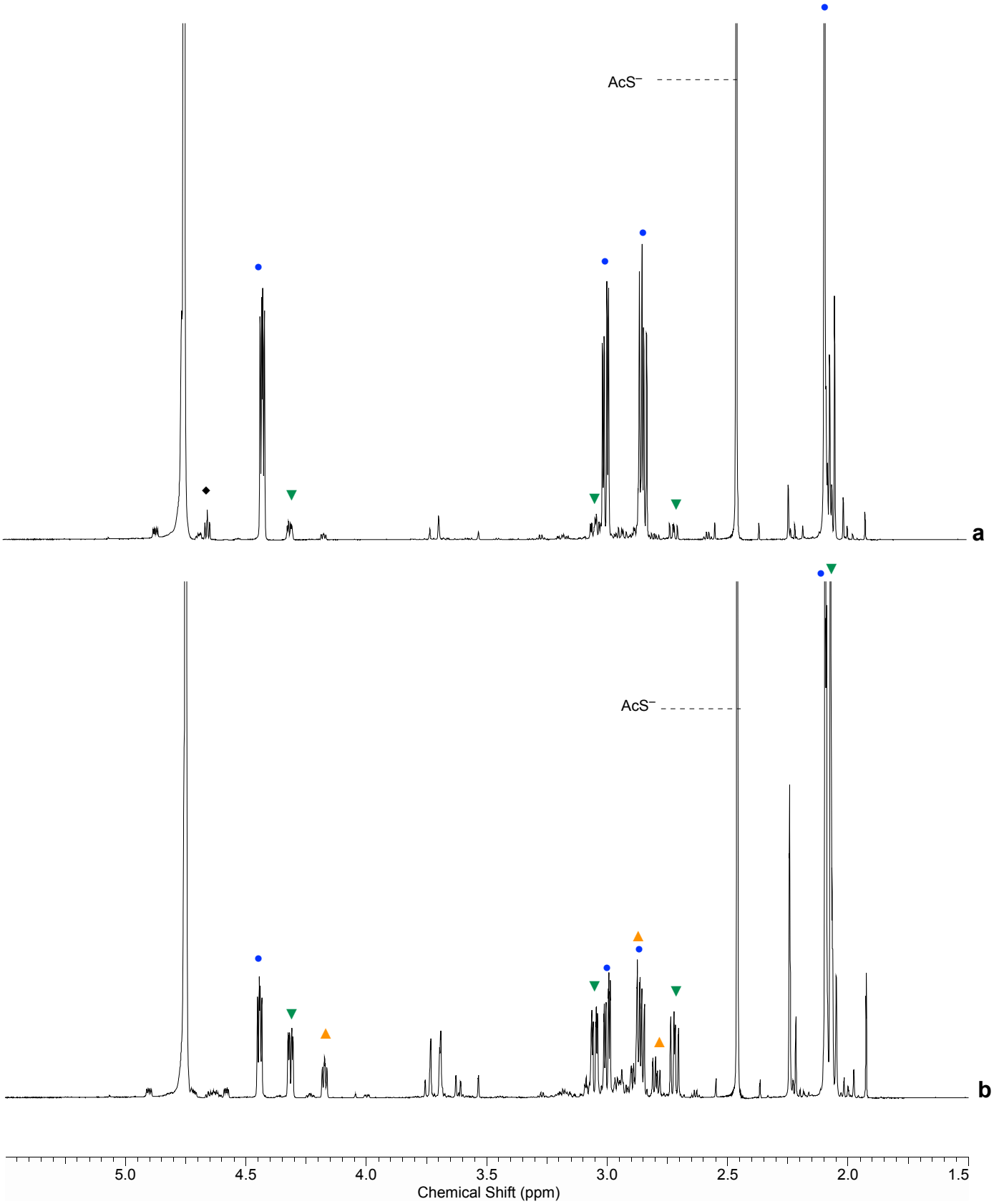


Fig. S47. ^{1}H NMR (700 MHz, H_2O, noesygppr1d, 1.50–5.50 ppm) to show the reaction of **Ac-Cys^{Ac}-CN** (50 mM) with NaSH (500 mM) in water after a) 16 h, and b) 6 d.

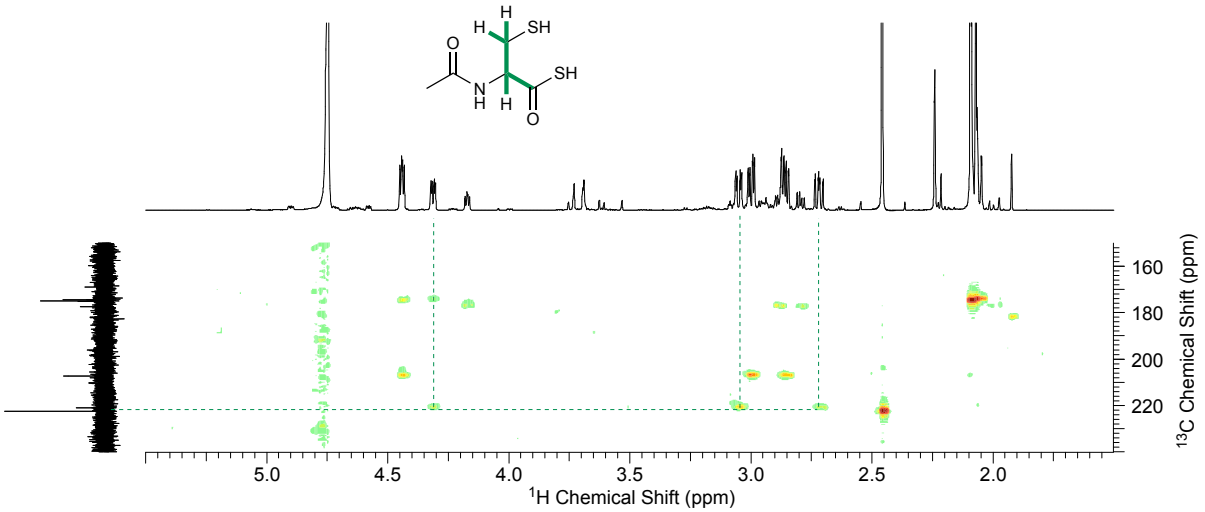
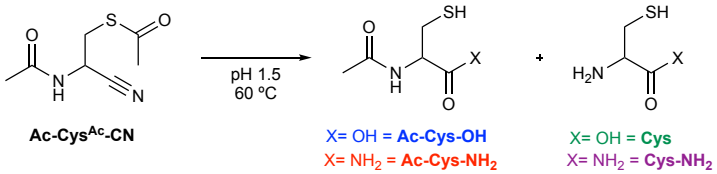


Fig. S48. ^{1}H–^{13}C HMBC (^{1}H–700 MHz [1.50–5.50 ppm], ^{13}C–176 MHz [150–240 ppm], H_2O) spectrum showing the diagnostic $^{2}J_{CH}$ coupling of Cys-(C2)–H at 4.32 ppm, and $^{3}J_{CH}$ coupling of Cys-(C3)–H and Cys-(C3)–H′ at 3.05 ppm and 2.72 ppm, with a thiocarbonyl resonance at 220.8 ppm. See Fig. S47 for expanded and labelled ^{1}H NMR spectrum.

Hydrolysis of N,S-diacetyl-DL-cysteine nitrile



N,*S*-Diacetyl-DL-cysteine nitrile **Ac-Cys^{Ac}-CN** (50 mM) was dissolved in water and adjusted to pH 1.5 by addition of 4 M HCl. The resulting solution was heated at 60 °C for 2 d and then analysed by NMR spectroscopy. A mixture of DL-cysteine **Cys** (7%), DL-cysteinamide **Cys-NH$_2$** (10%), *N*-acetyl-DL-cysteine **Ac-Cys-OH** (26%), and *N*-acetyl-DL-cysteinamide **Ac-Cys-NH$_2$** (27%) was observed. *N*-Acetyl-DL-cysteine (**Ac-Cys-OH**) and DL-cysteine (**Cys**) were authenticated by sample spiking with authentic commercial standards.

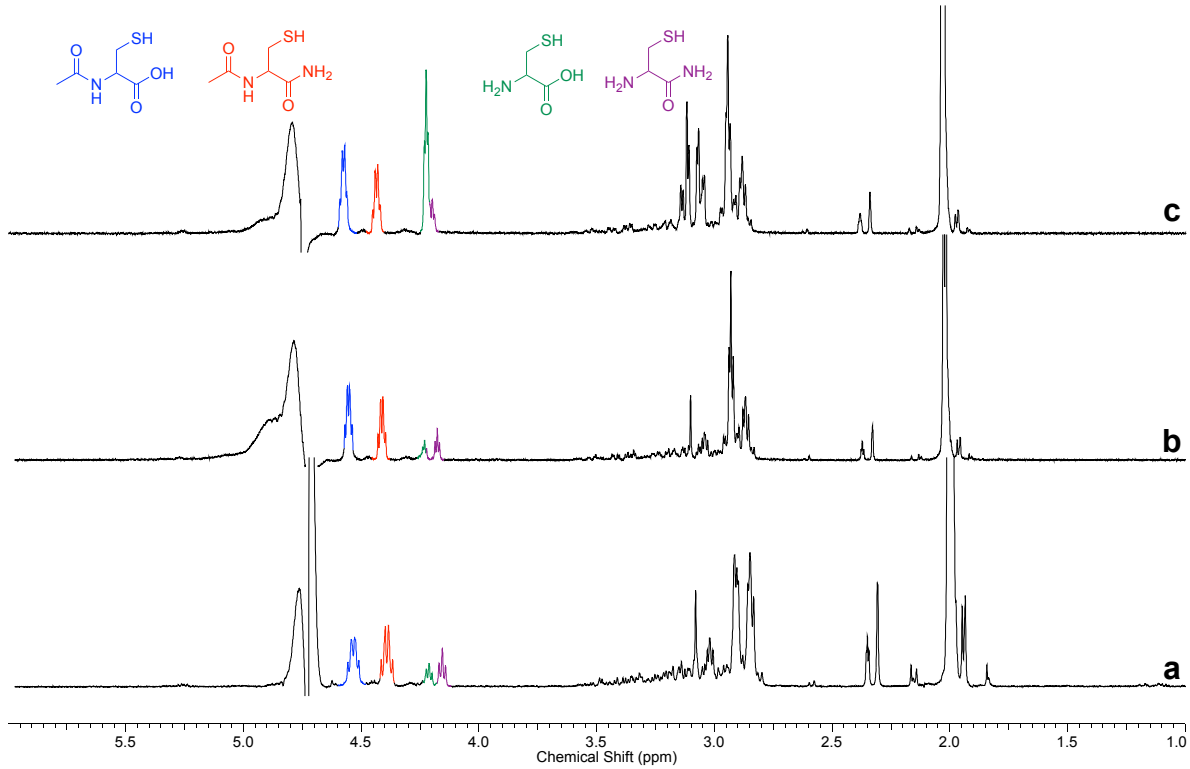
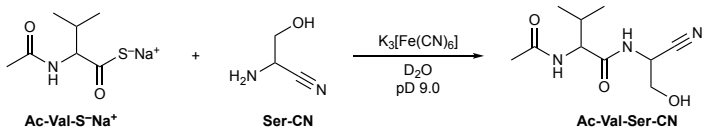


Fig. S49. ^{1}H NMR spectra (400 MHz, H_2O, noesygppr1d, 1.00–6.00 ppm) to show: **a**) the incubation of **Ac-Cys^{Ac}-CN** (50 mM) at pH 1.5 and 60 °C after 2 d; **b**) spike with commercial *N*-acetyl-L-cysteine (**Ac-Cys-OH**); **c**) spiking with commercial L-cysteine (**Cys**).

Synthesis of N-acetyl-DL-valinyl-DL-serine nitrile



Ac-Val-S⁻Na⁺ **Ser-CN** **Ac-Val-Ser-CN**

Sodium DL-2-acetamido-3-methylbutanethioate (*4*) **Ac-Val-S⁻Na⁺** (9.9 mg; 0.05 mmol) and DL-serine nitrile **Ser-CN** 8.6 mg; 0.10 mmol) were dissolved in D_2O (1 mL). The solution was adjusted to pD 9.0 with 4M NaOH. Potassium hexacyanoferrate (III) (49 mg; 0.15 mmol) was added and the reaction was stirred for 20 min. The reaction was concentrated *in vacuo*. The residue was triturated with dichloromethane (3 × 5 mL). The dichloromethane layers were concentrated *in vacuo* and the residue purified by flash column chromatography (SiO_2; EtOAc:MeOH (100:0→80:20) to give a diastereoisomeric mixture of *N*-acetyl-DL-valinyl-DL-serine nitrile **Ac-Val-Ser-CN** (9.1 mg; 80%) as a white solid. ^{1}H NMR (700 MHz, D_2O, noesygppr1d) (2 diastereoisomers) δ 4.90 (m, 2H, Ser-(C2)–H), 4.07 (d, *J* = 7.0 Hz, 1H, Val-(C2)–H), 4.07 (d, *J* = 7.2 Hz, 1H, Val-(C2)–H), 3.90 - 3.81 (m, 4H, Ser-(C3)–H_2), 2.11 - 2.04 (m, 2H, Val-(C3)–H), 2.03 (s, 3H, $COCH_3$), 2.02 (s, 3H, $COCH_3$), 0.96 - 0.91 (m, 12H, Val-(C4)–H_3, Val-(C4′)–H_3). ^{13}C NMR (176 MHz, D_2O) (2 diastereoisomers) δ 175.1 (**C**OCH_3), 175.1 (**C**OCH_3), 174.4 (Val-(C1)), 174.4 (Val-(C1)), 118.0 (Ser-(C1)), 118.0 (Ser-(C1)), 61.0 (Ser-(C3)), 61.0 (Ser-(C3)), 60.2 (Val-(C2)), 60.1 (Val-(C2)), 43.6 (2 × Ser-(C2)), 30.7 (Val-(C3)), 30.5 (Val-(C3)), 22.1 (2 × CO**C**H_3), 18.8 (Val-(C4)), 18.7 (Val-(C4)), 18.2 (Val-(C4′)), 18.1 (Val-(C4′)). HRMS-ESI $[M+H]^+$ calculated for $C_{10}H_{10}N_3O_3$ 228.1343; observed 228.1343. IR (cm^{-1}): 3283, 2964, 2937, 2410, 1633, 1540.

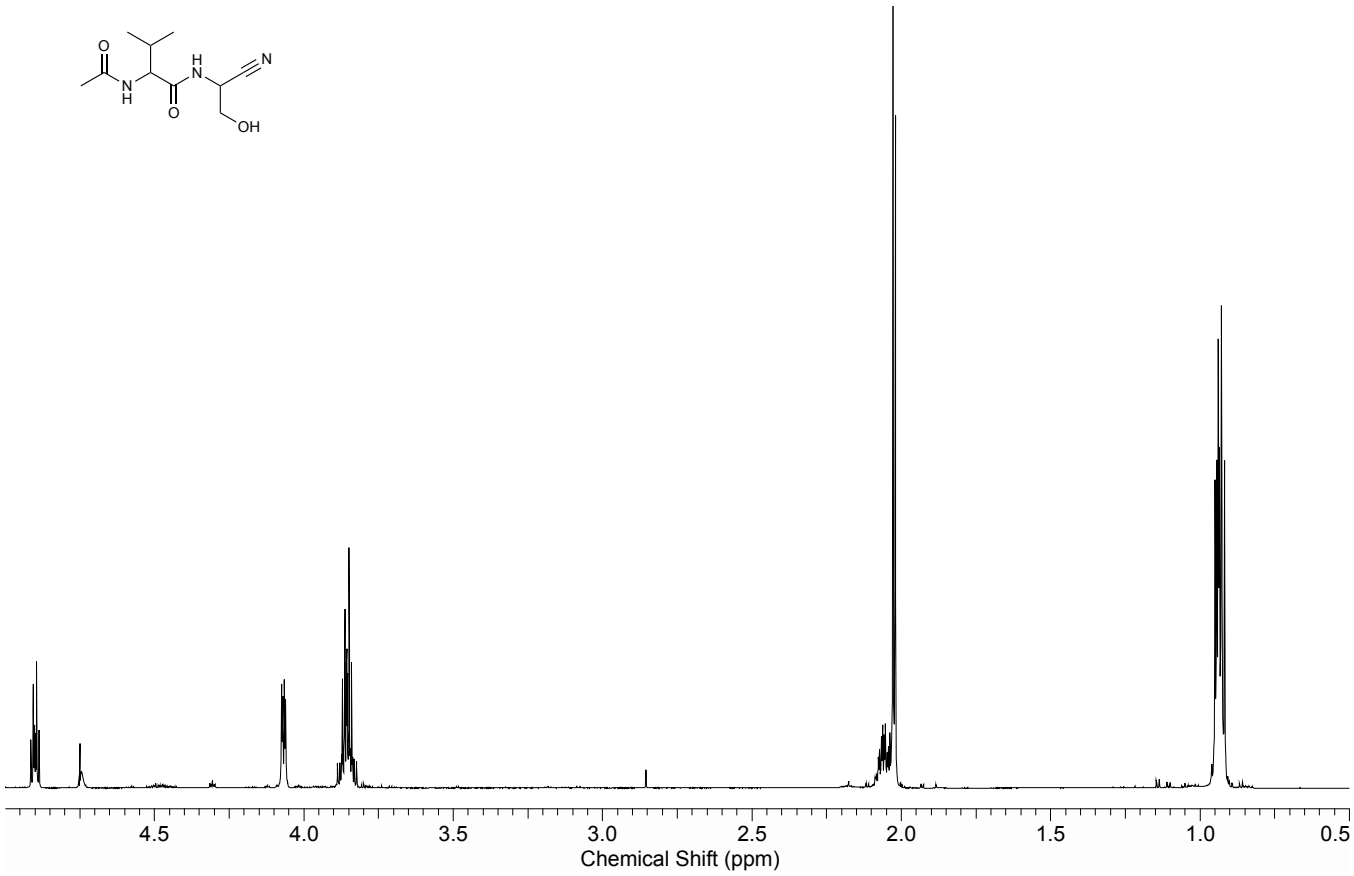


Fig. S50. ^{1}H NMR (700 MHz, D_2O, 0.5–5.0 ppm, noesygppr1d) spectrum to show *N*-acetyl-DL-valinyl-DL-serine nitrile (**Ac-Val-Ser-CN**).

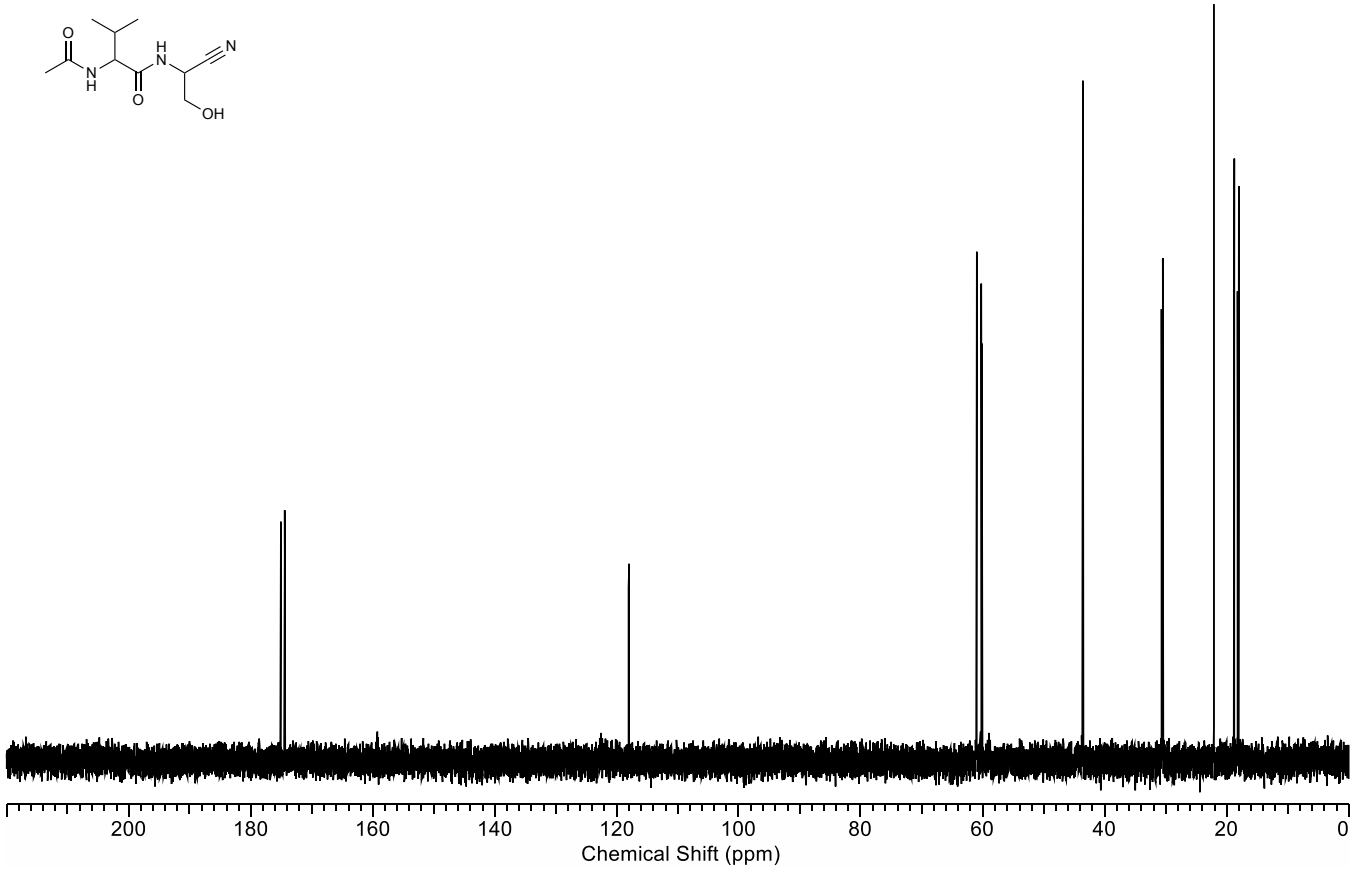
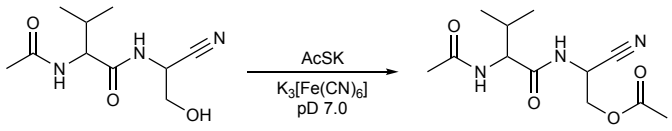


Fig. S51. ^{13}C NMR (176 MHz, D_2O, 0–220 ppm) spectrum to show *N*-acetyl-DL-valinyl-DL-serine nitrile (**Ac-Val-Ser-CN**).

Acetylation of N-acetyl-DL-valinyl-DL-serine nitrile with thioacetate



Ac-Val-Ser-CN **Ac-Val-Ser^{Ac}-CN**

A solution of *N*-acetyl-DL-valinyl-DL-serine nitrile **Ac-Val-Ser-CN** (25 mM; 5.7 mg, 0.025 mmol) and potassium thioacetate (**AcSK**; 28.6 mg, 0.25 mmol) in D_2O (1 mL) was adjusted to pD 7.0 with HCl/NaOH. Potassium hexacyanoferrate(III) ($K_3[Fe(CN)_6]$; 164 mg, 0.50 mmol) was added and the reaction was stirred rapidly whilst maintaining the solution at pD 7 with 0.5 M NaOH. After 1 h the reaction was centrifuged and the supernatant analysed by NMR spectroscopy. Complete conversion of **Ac-Val-Ser-CN** to a diastereoisomeric mixture of *N,O*-diacetyl-DL-valinyl-DL-serine nitrile **Ac-Val-Ser^{Ac}-CN** was confirmed by the disappearance of the Ser-(C3)–H_2 of **Ac-Val-Ser-CN** (Fig. S52.) The reaction mixture was lyophilised and the residue purified by flash column chromatography (SiO_2; EtOAc:MeOH (100:0→80:20) to give a mixture of diastereoisomers of *N,O*-diacetyl-DL-valinyl-DL-serine nitrile **Ac-Val-Ser^{Ac}-CN** and *N*-acetyl-DL-valinyldehydroalanine nitrile **Ac-Val-Dha-CN** (combined isolated material = 5.2 mg; >77%) (Fig. S53–Fig. S57) .

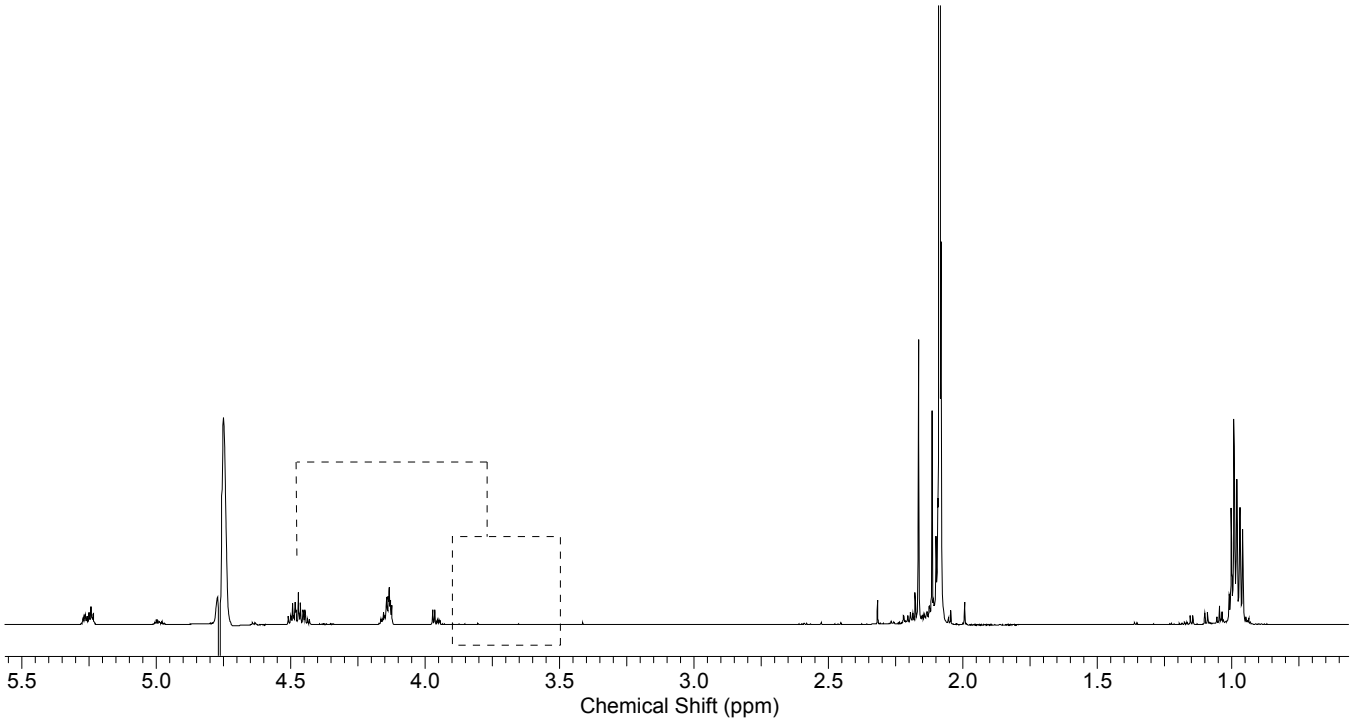


Fig. S52. ^{1}H NMR (700 MHz, D_2O, 0.5–5.5 ppm, noesygppr1d) spectrum to show a diastereoisomeric mixture of *N,O*-diacetyl-DL-valinyl-DL-serine nitrile **Ac-Val-Ser^{Ac}-CN** obtained after the reaction of *N*-acetyl-DL-valinyl-DL-serine nitrile **Ac-Val-Ser-CN** (25 mM) with potassium thioacetate (250 mM) and $K_3[Fe(CN)_6]$ at pD 7.0. The disappearance of Ser-(C3)–H_2 of **Ac-Val-Ser-CN** in the 3.50-4.00 ppm region upon (C3)–OH acetylation results in a discernable lower field resonance of the (C3)–H_2 region of **Ac-Val-Ser^{Ac}-CN** at 4.42–4.52 ppm, which is highlighted with the dotted box and lines.

Data for N,O-Diacetyl-DL-valinyl-DL-serine nitrile ***Ac-Val-Ser^{Ac}-CN****–(A) (diastereoisomer A (major diastereoisomer in Fig. S53))*

^{1}H NMR (700 MHz, D_2O) δ 5.17 (dd, *J* = 5.0, 5.8 Hz, 1H, Ser-(C2)–H), 4.42 (ABX, *J* = 5.8, 11.4 Hz, 1H, Ser-(C3)–H), 4.38 (ABX, *J* = 5.0, 11.4 Hz, 1H, Ser-(C3)–H)′), 4.04 (d, *J* = 7.1 Hz, 1H, Val-(C2)–H), 2.12 (s, 3H, Ser-$COCH_3$), 2.09 - 2.03 (m, 1H, Val-(C3)–H), 2.02 (s, 3H, Val-$COCH_3$), 0.95 (d, *J* = 7.1 Hz, 3H, Val-(C4)–H_3), 0.94 (d, *J* = 7.1 Hz, 3H, Val-(C4)–H_3′). ^{13}C NMR (176 MHz, D_2O) δ 175.01 (Val-**C**OCH_3), 174.4 (Val-(C1)), 173.7, (Ser-**C**OCH_3), 117.0 (Ser-(C1)), 62.8 (Ser-(C3)), 60.1 (Val-(C2)), 40.6 (Ser-(C2)), 30.6 (Val-(C3)), 22.1 (Val-CO**C**H_3), 20.6 (Ser-CO**C**H_3), 18.7 (Val-(C4)), 18.2 (Val-(C4′)). HRMS-ESI for **Ac-Val-Ser^{Ac}-CN** $[M+H]^+$ calculated for $C_{12}H_{20}N_3O_4$ 270.1448; observed 270.1448. IR (cm^{-1}): 3280, 3053, 2965, 2418, 1632, 1539.

Data for N,O-Diacetyl-DL-valinyl-DL-serine nitrile ***Ac-Val-Ser^{Ac}-CN****–(B) (diastereoisomer B (minor diastereoisomer in Fig. S53))*

^{1}H NMR (700 MHz, D_2O) δ 5.20 (dd, *J* = 4.9, 6.0 Hz, 1H, Ser-(C2)–H), 4.42 (ABX, *J* = 6.0, 11.4 Hz, 1H, Ser-(C3)–H), 4.39 - 4.36 (obs., 1H, Ser-(C3)–H)′), 4.05 (d, *J* = 7.0 Hz, 1H, Val-(C2)–H), 2.12 (s, 3H, Ser-$COCH_3$), 2.08 - 2.03 (m, 1H, Val-(C3)–H), 2.03 (s, 3H, Val-$COCH_3$), 0.94 (d, *J* = 7.0 Hz, 3H, Val-(C4)–H_3), 0.92 (d, *J* = 7.0 Hz, 3H, Val-(C4)–H_3′). ^{13}C NMR (176 MHz, D_2O) δ 175.03 (Val-**C**OCH_3), 174.3 (Val-(C1)), 173.7, (Ser-**C**OCH_3), 116.9 (Ser-(C1)), 62.9 (Ser-(C3)), 60.2 (Val-(C2)), 40.6 (Ser-(C2)), 30.5 (Val-(C3)), 22.1 (Val-CO**C**H_3), 20.6 (Ser-CO**C**H_3), 18.7 (Val-(C4)), 18.1 (Val-(C4′)).

Data for N,O-Diacetyl-DL-valinyl-DL-serine nitrile ***Ac-Val-Ser^{Ac}-CN****–(B) (Fig. S56)*

^{1}H NMR (700 MHz, D_2O) δ 5.20 (dd, *J* = 4.9, 5.9 Hz, 1H, Ser-(C2)–H), 4.42 (ABX, *J* = 5.9, 11.4 Hz, 1H, Ser-(C3)–H), 4.37 (ABX, *J* = 4.9, 11.4 Hz, 1H, Ser-(C3)–H)′), 4.05 (d, *J* = 7.0 Hz, 1H, Val-(C2)–H), 2.12 (s, 3H, Ser-$COCH_3$), 2.07 - 2.03 (m, 1H, Val-(C3)–H), 2.02 (s, 3H, Val-$COCH_3$), 0.93 (d, *J* = 7.0 Hz, 3H, Val-(C4)–H_3), 0.92 (d, *J* = 7.0 Hz, 3H, Val-(C4)–H_3′. ^{13}C NMR (176 MHz, D_2O) δ 175.0 (Val-**C**OCH_3), 174.3 (Val-(C1)), 173.8, (Ser-**C**OCH_3), 116.9 (Ser-(C1)), 62.9 (Ser-(C3)), 60.2 (Val-(C2)), 40.6 (Ser-(C2)), 30.5 (Val-(C3)), 22.1 (Val-CO**C**H_3), 20.6 (Ser-CO**C**H_3), 18.7 (Val-(C4)), 18.1 (Val-(C4′)).

Data for N-acetyl-DL-valinyldehydroalanine nitrile ***Ac-Val-Dha-CN*** *(Fig. S56)*

^{1}H NMR (700 MHz, D_2O, partial data) δ 5.92 (d, *J* = 1.8 Hz, 1H, Dha-(C3)–H), 5.83 (d, *J* = 1.8 Hz, 1H, 1H, Dha-(C3)–H′), 2.05 – 2.03 (m,1H, Val-(C3)–H), 2.03 (s, 3H, $COCH_3$), 0.96 (d, *J* = 4.7 Hz, 3H, Val-(C4)–H), 0.95 (d, *J* = 4.7 Hz, 3H, Val-(C4)–H′). ^{13}C NMR (176 MHz, D_2O) δ 175.1 (CO), 173.3 (CO), 122.8 (Dha-(C3)), 115.6 (Dha-(C2)), 115.1 (Dha-(C1)), 60.4 (Val-(C2)), 30.6 (Val-(C3)), 22.1 (CO**C**H_3), 18.7 (Val-(C4)), 18.1 (Val-(C4′)).

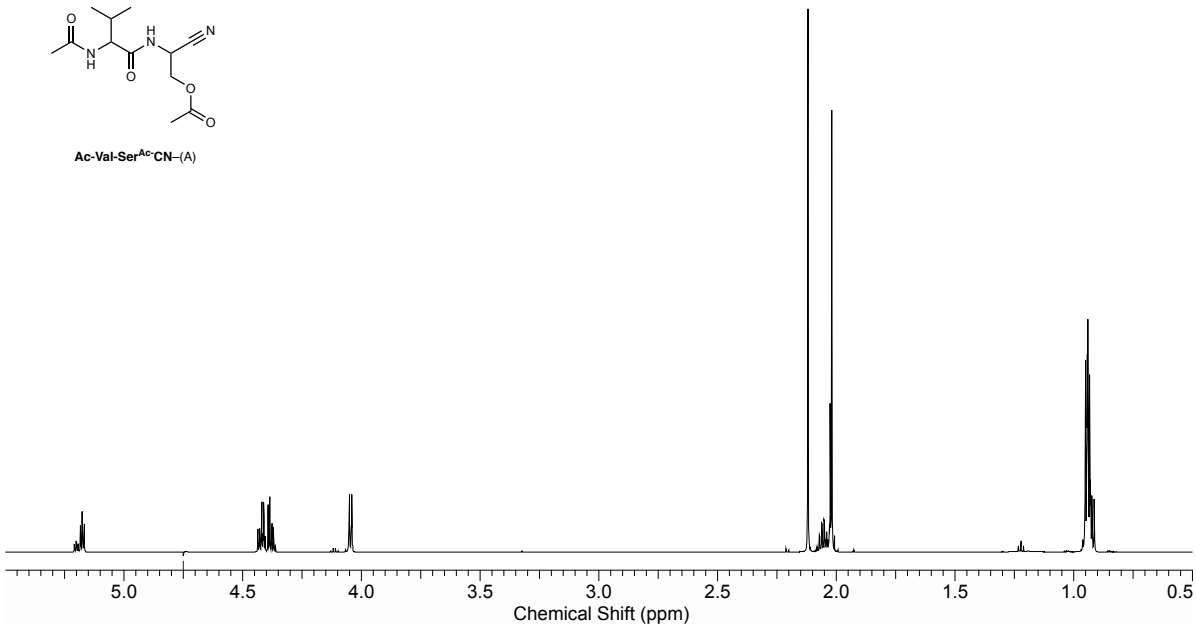


Fig. S53. ^{1}H NMR (700 MHz, D_2O, 0.5–5.2 ppm, noesygppr1d) spectrum to show a diastereoisomeric mixture of *N,O*-diacetyl-DL-valinyl-DL-serine nitrile **Ac-Val-Ser^{Ac}-CN** (**Ac-Val-Ser^{Ac}-CN**–(A): **Ac-Val-Ser^{Ac}-CN**–(B); 75:25) after flash chromatography of the crude mixture obtained from the reaction of **Ac-Val-Ser-CN** with thioacetate and $K_3[Fe(CN)_6]$ at pD 7.0.

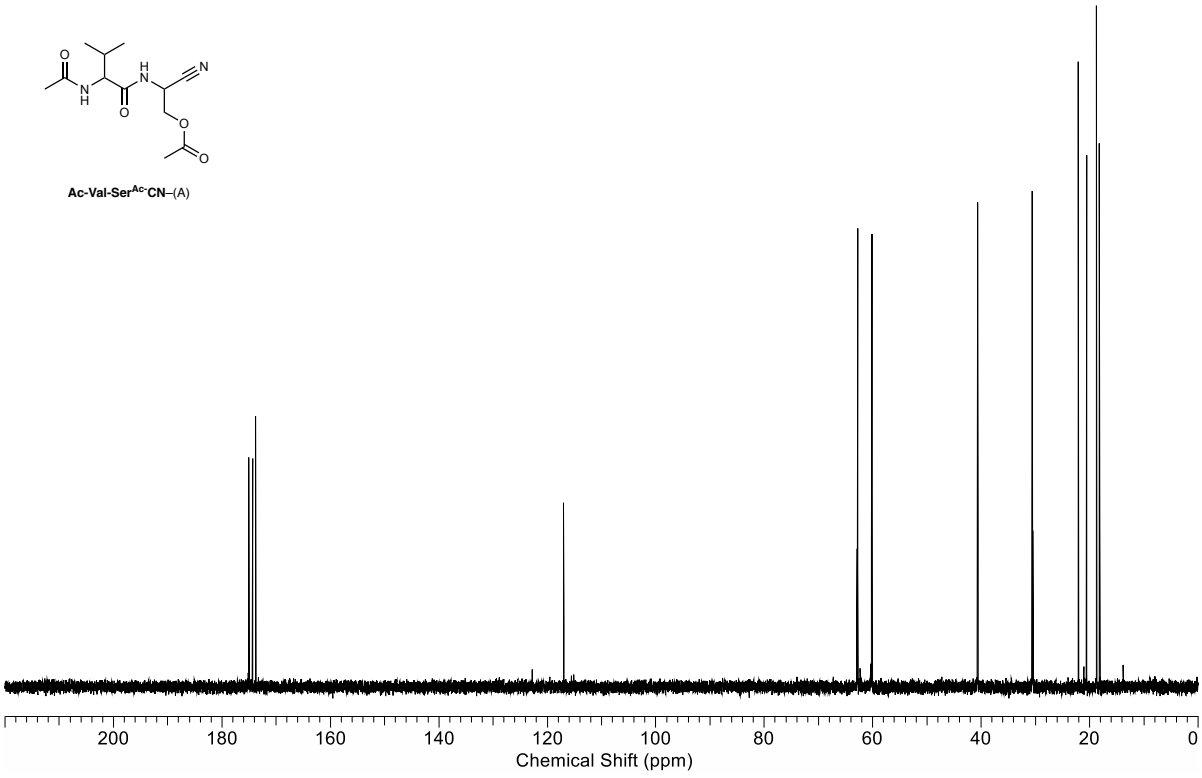


Fig. S54. ^{13}C NMR (176 MHz, D_2O, 0–220 ppm, noesygppr1d) spectrum to show a diastereoisomeric mixture of *N,O*-diacetyl-DL-valinyl-DL-serine nitrile **Ac-Val-Ser^{Ac}-CN** (**Ac-Val-Ser^{Ac}-CN**–(A): **Ac-Val-Ser^{Ac}-CN**–(B)) after flash chromatography of the crude mixture obtained from the reaction of **Ac-Val-Ser-CN** with thioacetate and $K_3[Fe(CN)_6]$ at pD 7.0.

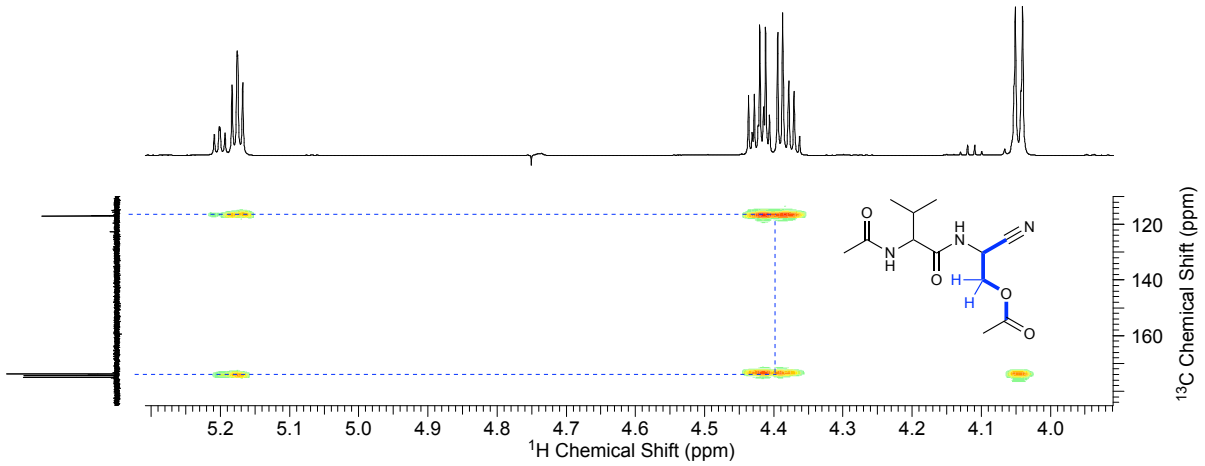


Fig. S55. ^{1}H–^{13}C HMBC (^{1}H–700 MHz [3.9–5.3 ppm], ^{13}C–176 MHz [110–185 ppm], D_2O) spectrum showing diagnostic $^{3}J_{CH}$ couplings of Ser-(C3)–H and Ser-(C3)–H′ of both diastereoisomers of **Ac-Val-Ser^{Ac}-CN** between 4.36 and 4.42 ppm with Ser-C1 (CN) resonances at 117.0 ppm (major diastereoisomer A, **Ac-Val-Ser^{Ac}-CN**–(A)) and 116.9 ppm (minor diastereoisomer B, **Ac-Val-Ser^{Ac}-CN**–(B)), and at 173.7 ppm for the C=O resonance of Ser-**C**OCH_3. See Fig. S54 for the ^{1}H NMR spectrum.

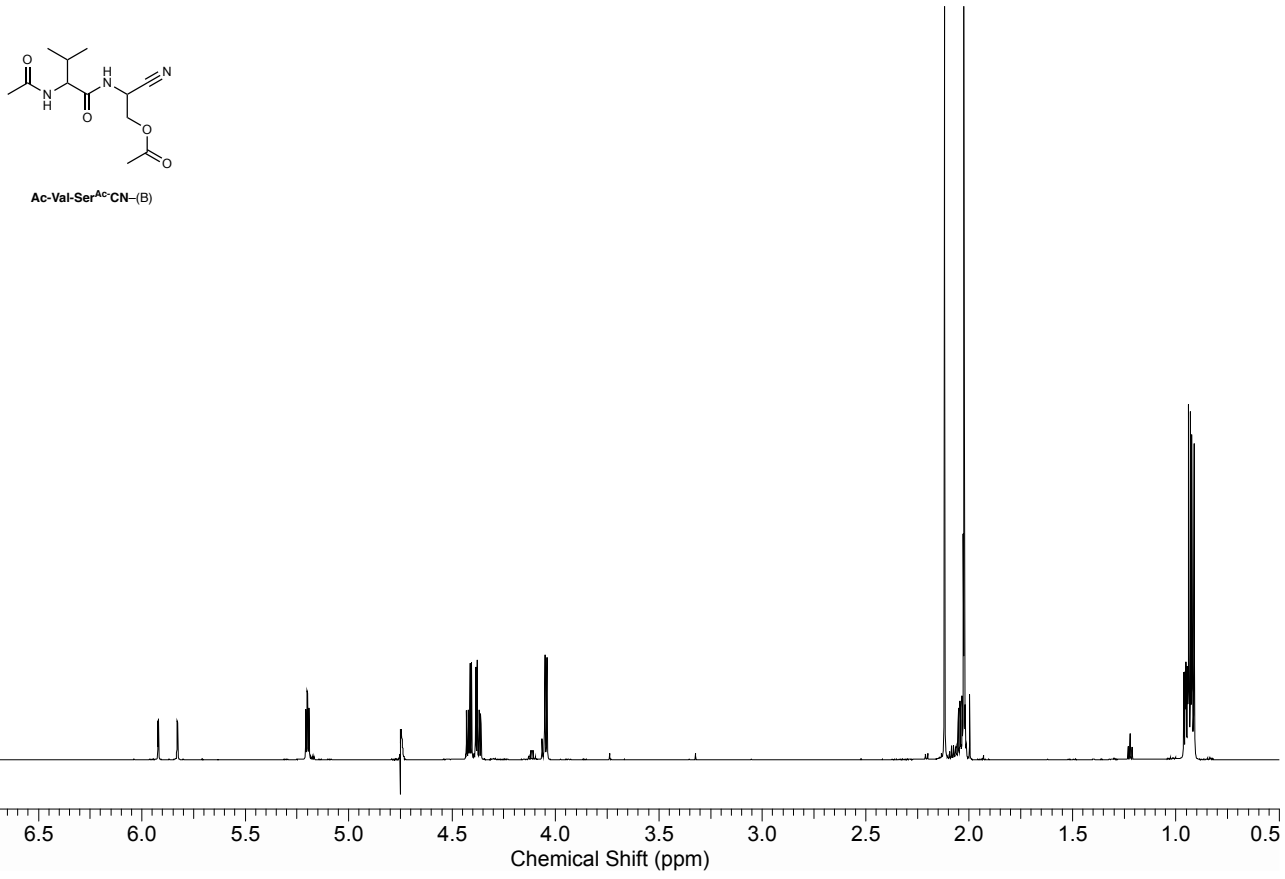


Fig. S56. ^{1}H NMR (700 MHz, D_2O, 0.5–6.0 ppm, noesygppr1d) spectrum to show the minor diastereoisomer B *N,O*-diacetyl-DL-valinyl-DL-serine nitrile **Ac-Val-Ser^{Ac}-CN**–(B) and *N*-acetyl-DL-valinyldehydroalanine nitrile **Ac-Val-Dha-CN** after flash chromatography of the crude mixture obtained from the reaction of **Ac-Val-Ser-CN** with thioacetate and $K_3[Fe(CN)_6]$ at pD 7.0.

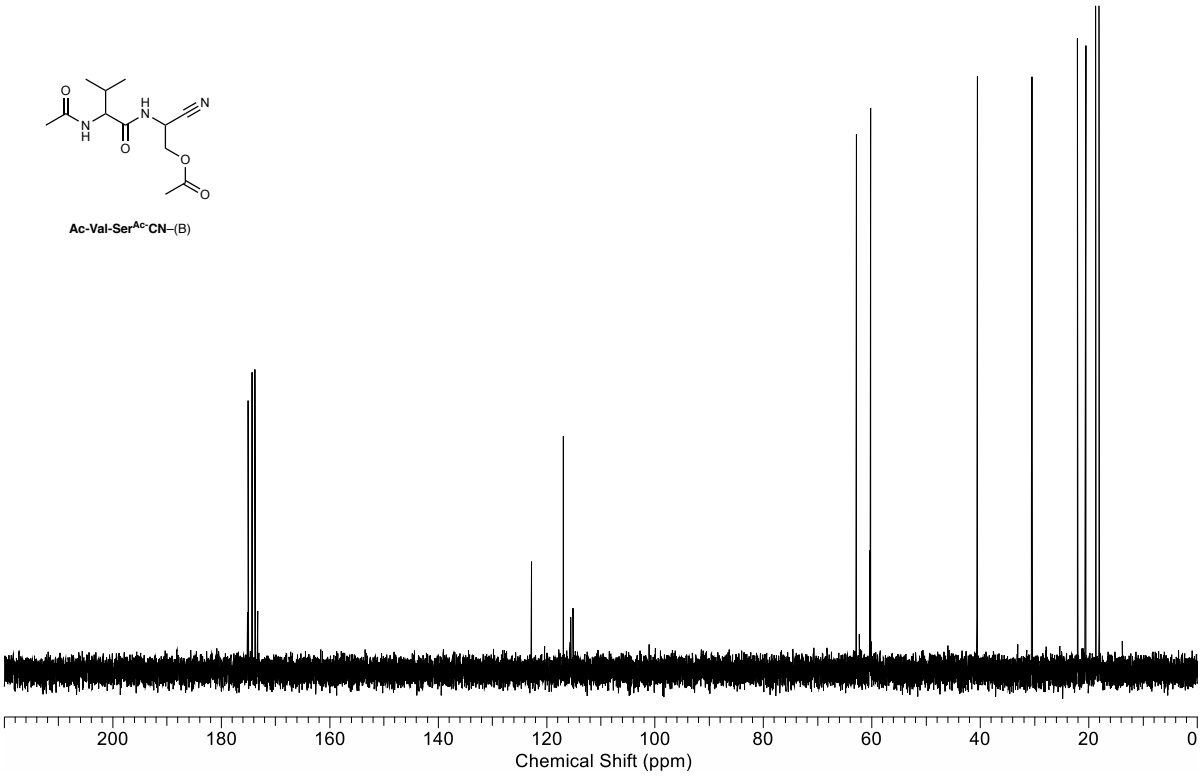
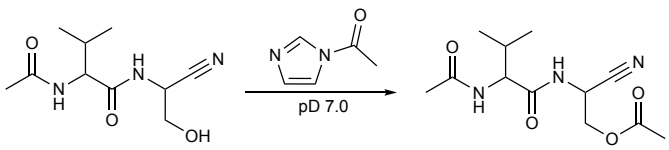


Fig. S57. ^{13}C NMR (176 MHz, D_2O, 0–220 ppm, noesygppr1d) spectrum to show the minor diastereoisomer B *N,O*-diacetyl-DL-valinyl-DL-serine nitrile **Ac-Val-Ser^{Ac}-CN**–(B) and *N*-acetyl-DL-valinyldehydroalanine nitrile **Ac-Val-Dha-CN** after flash chromatography of the crude mixture obtained from the reaction of **Ac-Val-Ser-CN** with thioacetate and $K_3[Fe(CN)_6]$ at pD 7.0.

Acetylation of N-acetyl-DL-valinyl-DL-serine nitrile with N-acetylimidazole



Ac-Val-Ser-CN **Ac-Val-Ser^{Ac}-CN**

A solution of *N*-acetyl-DL-valinyl-DL-serine nitrile **Ac-Val-Ser-CN** (100 mM; 22.7 mg, 0.10 mmol) and *N*-acetyl imidazole (55 mg, 0.50 mmol) was adjusted to pH 7. The acetylation of **Ac-Val-Ser-CN** (>95%) to give a diastereoisomeric mixture of **Ac-Val-Ser^{Ac}-CN** (**Ac-Val-Ser^{Ac}-CN**–(A): **Ac-Val-Ser^{Ac}-CN**–(B); 62:38).) was observed after 2 h (Fig. S58).

*Data for N,O-diacetyl-DL-valinyl-DL-serine nitrile **Ac-Val-Ser^{Ac}-CN**–(A) (major diastereoisomer A in Fig. S58)*

^{1}H NMR (700 MHz, D_2O) δ 5.07 (dd, *J* = 5.2, 5.8 Hz, 1H, Ser-(C2)–H), 4.33 (ABX, *J* = 5.8, 11.4 Hz, 1H, Ser-(C3)–H), 4.28 (ABX, *J* = 5.2, 11.4 Hz, 1H, Ser-(C3)–H)′), 3.95 (d, *J* = 7.2 Hz, 1H, Val-(C2)–H), 2.02 (s, 3H, Ser-$COCH_3$), 1.99 - 1.94 (m, 1H, Val-(C3)–H), 1.93 (s, 3H, Val-$COCH_3$), 0.85 (d, *J* = 7.2 Hz, 3H, Val-(C4)–H_3), 0.84 (d, *J* = 7.2 Hz, 3H, Val-(C4)–H_3′).

*Data for N,O-diacetyl-DL-valinyl-DL-serine nitrile **Ac-Val-Ser^{Ac}-CN**–(B) (major diastereoisomer A in Fig. S58)*

^{1}H NMR (700 MHz, D_2O) δ 5.10 (dd, *J* = 5.0, 5.9 Hz, 1H, Ser-(C2)–H), 4.33 (ABX, *J* = 5.9, 11.4 Hz, 1H, Ser-(C3)–H), 4.28 (ABX, *J* = 5.0, 11.4 Hz, 1H, Ser-(C3)–H)′), 3.95 (d, *J* = 7.2 Hz, 1H, Val-(C2)–H), 2.02 (s, 3H, Ser-$COCH_3$), 1.99 - 1.94 (m, 1H, Val-(C3)–H), 1.93 (s, 3H, Val-$COCH_3$), 0.85 – 0.84 (obs., 3H, Val-(C4)–H_3), 0.82 (d, *J* = 7.2 Hz, 3H).

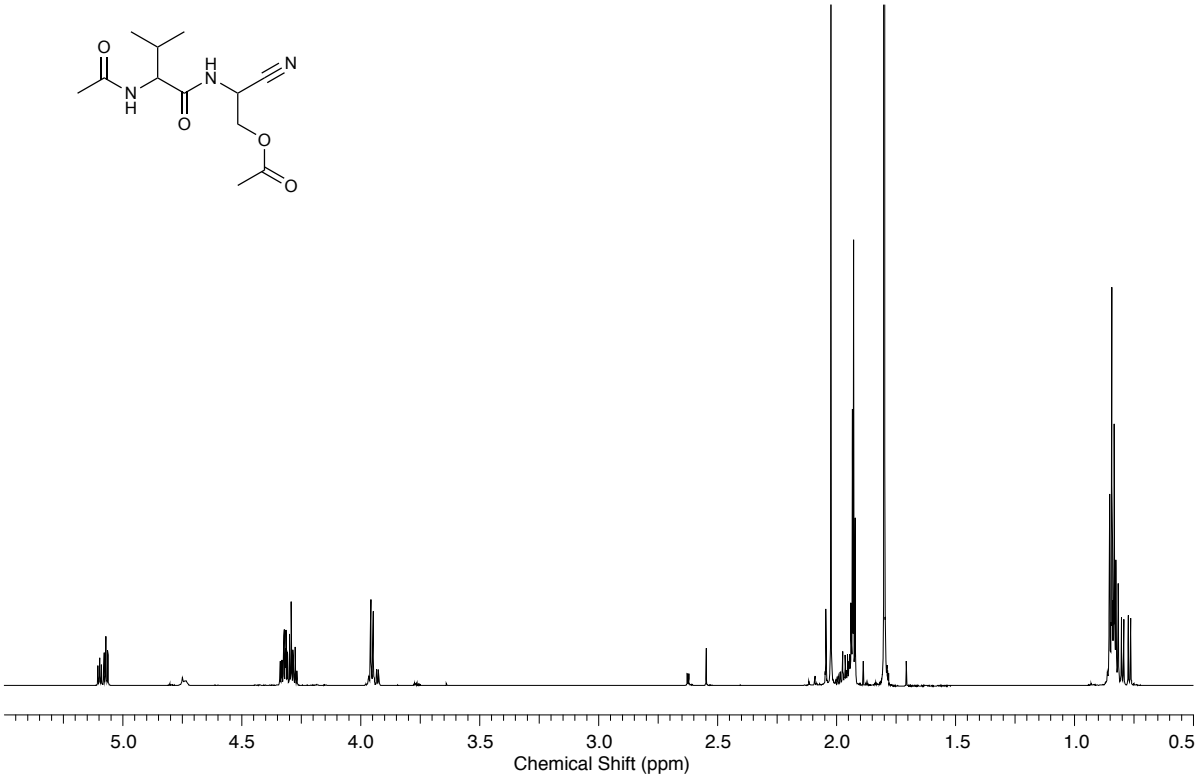


Fig. S58. ^{1}H NMR (700 MHz, D_2O, 0.5–5.2 ppm, noesygppr1d) spectrum to show a diastereoisomeric mixture of *N,O*-diacetyl-DL-valinyl-DL-serine nitrile **Ac-Val-Ser^{Ac}-CN** (**Ac-Val-Ser^{Ac}-CN**–(A): **Ac-Val-Ser^{Ac}-CN**–(B); 38:67) obtained after the reaction of *N*-acetyl-DL-valinyl-DL-serine nitrile **Ac-Val-Ser-CN** (100 mM) with *N*-acetylimidazole (5 equiv.) at pD 7.0 and room temperature after 2 h.

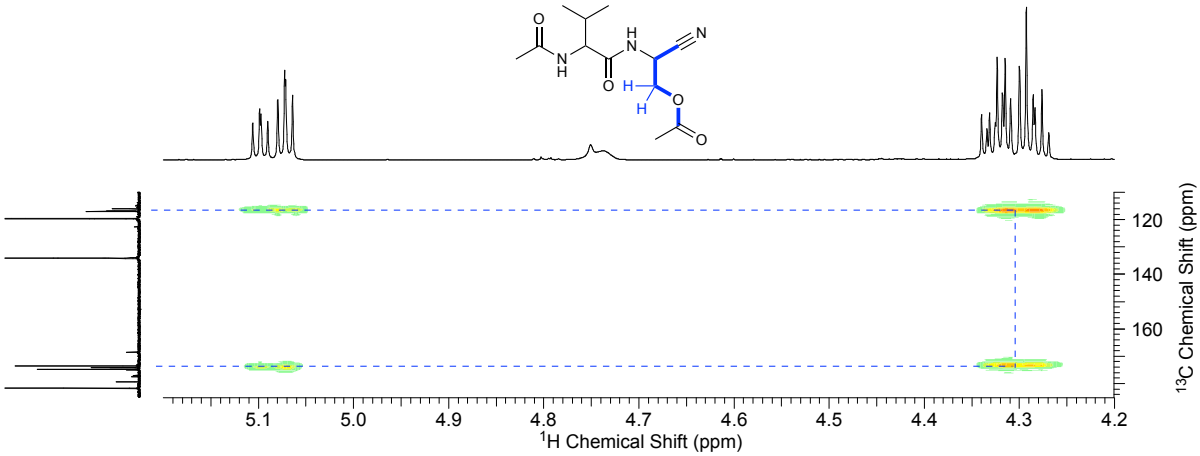
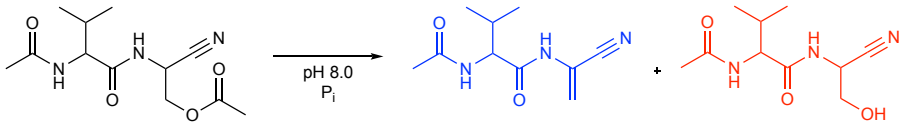


Fig. S59. ^{1}H–^{13}C HMBC (^{1}H–700 MHz [4.2–5.2 ppm], ^{13}C–176 MHz [110–185 ppm], D_2O) spectrum showing diagnostic $^{3}J_{CH}$ couplings of Ser-(C3)–H and Ser-(C3)–H′ of both diastereoisomers of **Ac-Val-Ser^{Ac}-CN** between 4.38 and 4.42 ppm with Ser-C1 (CN) resonances at 116.9 ppm (major diastereoisomer A) and 117.0 ppm (minor diastereoisomer B), and at 173.6 ppm for the C=O resonance of Ser-**C**OCH_3. See Fig. S58 for the ^{1}H NMR spectrum.

Synthesis of N-acetyl-DL-valinyldehydroalanine nitrile from N,O-diacetyl-DL-valinylserine nitrile in phosphate by acetic acid elimination



N,O-Diacetyl-DL-valinyl-DL-serine nitrile **Ac-Val-Ser^{Ac}-CN** (10 mM) was dissolved in phosphate buffer (500 mM) at pH 8. The reaction was incubated at room temperature and periodically analysed NMR spectroscopy. Conversion of **Ac-Val-Ser^{Ac}-CN** to a mixture of *N,O*-diacetyl-DL-valinyldehydroalanine nitrile **Ac-Val-Dha-CN** (90%) and *N*-acetyl-DL-valinyl-DL-serine nitrile **Ac-Val-Ser-CN** (10%) was observed after 4 d (Fig. S60).

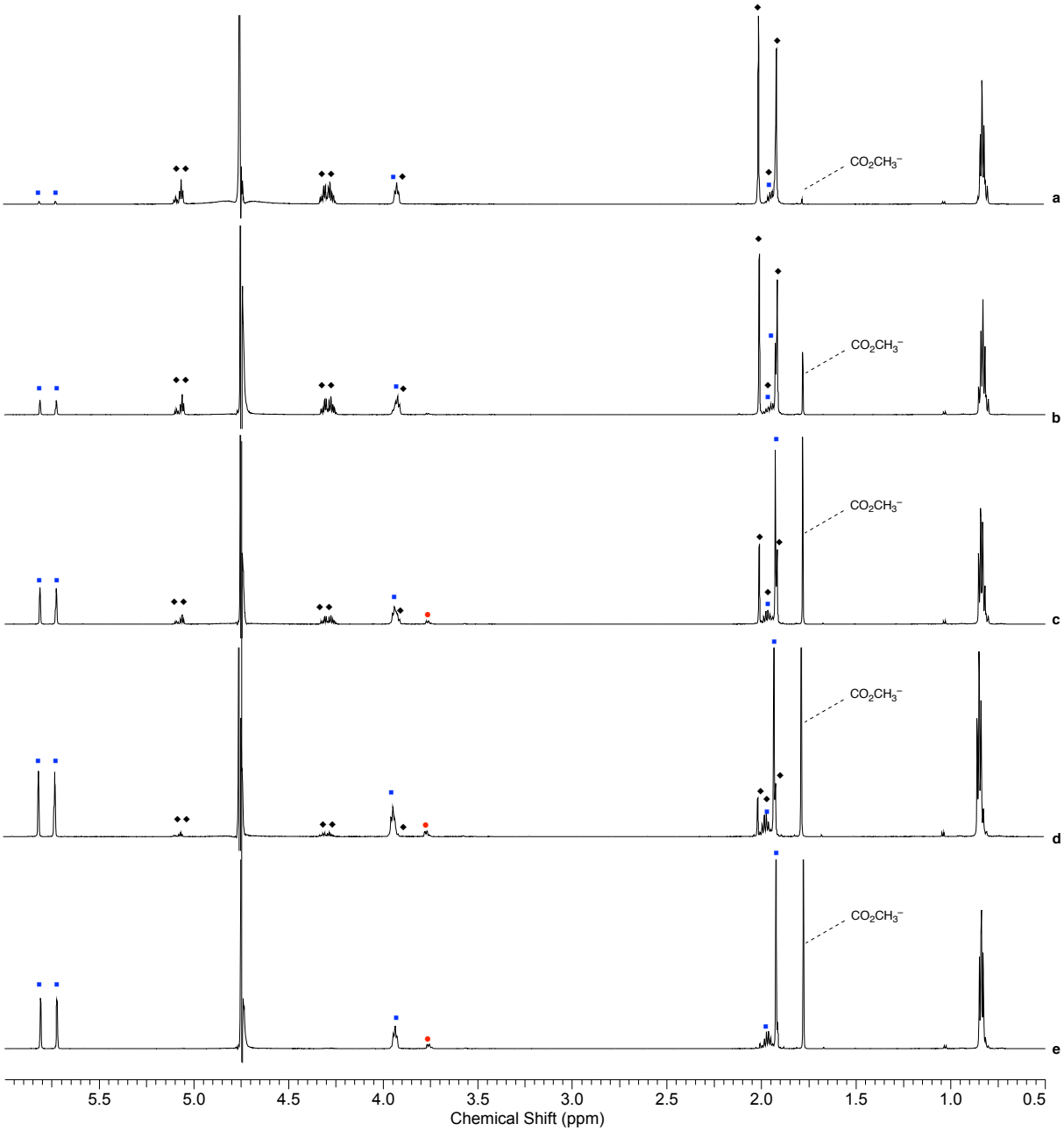
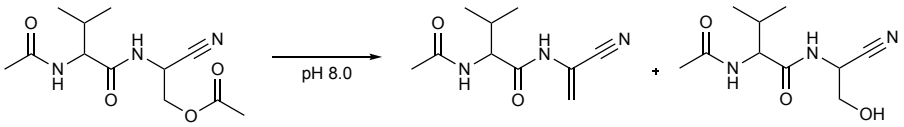


Fig. S60. ^{1}H NMR (600 MHz, H_2O, noesygppr1d, 0.50–6.00 ppm) to show conversion of **Ac-Val-Ser^{Ac}-CN** (10 mM) to **Ac-Val-Dha-CN** at room temperature in phosphate buffer (pH 8; 500 mM) after a) 30 mins; b) 7 h; c) 24 h; d) 48 h; e) 96 h.

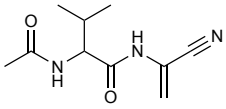
Synthesis of N-acetyl-DL-valinyldehydroalanine nitrile from N,O-diacetyl-DL-valinyl-DL-serine nitrile in water by acetic acid elimination



Ac-Val-Ser^{Ac}-CN **Ac-Val-Dha-CN** **Ac-Val-Ser-CN**

N,O-Diacetyl-DL-valinyl-DL-serine nitrile **Ac-Val-Ser^{Ac}-CN** (5.2 mg; 10 mM;) was dissolved in water (1.93 mL) at pH 8. The solution pH was monitored and periodically readjusted to pH 8 with 0.1 M NaOH. After 4 d the reaction composition was **Ac-Val-Dha-CN**:**Ac-Val-Ser^{Ac}-CN**:**Ac-Val-Ser-CN** 66:11:22.The reaction was lyophilised and the residue was purified by flash column chromatography (SiO_2; EtOAc (100%)) to give *N*-Acetyl-DL-valinyldehydroalanine nitrile **Ac-Val-Dha-CN** as a white gum (2.02 mg, 50%).

*Data for N-acetyl-DL-valinyldehydroalanine nitrile **Ac-Val-Dha-CN***



^{1}H NMR (700 MHz, D_2O) δ 5.92 (d, *J* = 1.8 Hz, 1H, Dha-(C3)–H), 5.83 (d, *J* = 1.8 Hz, 1H, Dha-(C3)–H′), 4.06 (d, *J* = 7.2 Hz, 1H, Val-(C2)–H), 2.08 (app. sxt, *J* = 6.9 Hz, 1H, Val-(C3)–H), 2.03 (s, 3H, $COCH_3$), 0.96 (d, *J* = 6.7 Hz, 3H, Val-(C3)–H_3), 0.95 (d, *J* = 7.0 Hz, 3H, Val-(C3)–H_3'). ^{13}C NMR (176 MHz, D_2O) δ 175.1 (**C**OCH_3), 173.3 (Val-(C1)), 122.8 (Dha-(C3)), 115.6 (Dha-(C2)), 115.1 (Dha-(C1), 60.4 (Val-(C2)), 30.6 (Val-(C3)), 22.1 (CO**C**H_3), 18.7 (Val-(C4)), 18.1 (Val-(C4′)). HRMS-ESI $[M+H^+]^+$ calculated for formula $C_{10}H_{16}N_3O_2^+$, 210.1241; found 210.1237. IR (cm^{-1}): 3283, 3033, 2972, 1687, 1644, 1627.

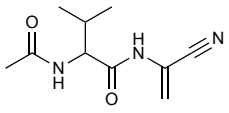


Fig. S61. ^{1}H NMR (700 MHz, D_2O, 0.5–7.0 ppm, noesygppr1d) spectrum to show a *N*-acetyl-DL-valinyldehydroalanine nitrile **Ac-Val-Dha-CN** after flash column chromatography of the crude mixture obtained from the incubation of **Ac-Val-Ser^{Ac}-CN** at pH 8.0 for 4 d.

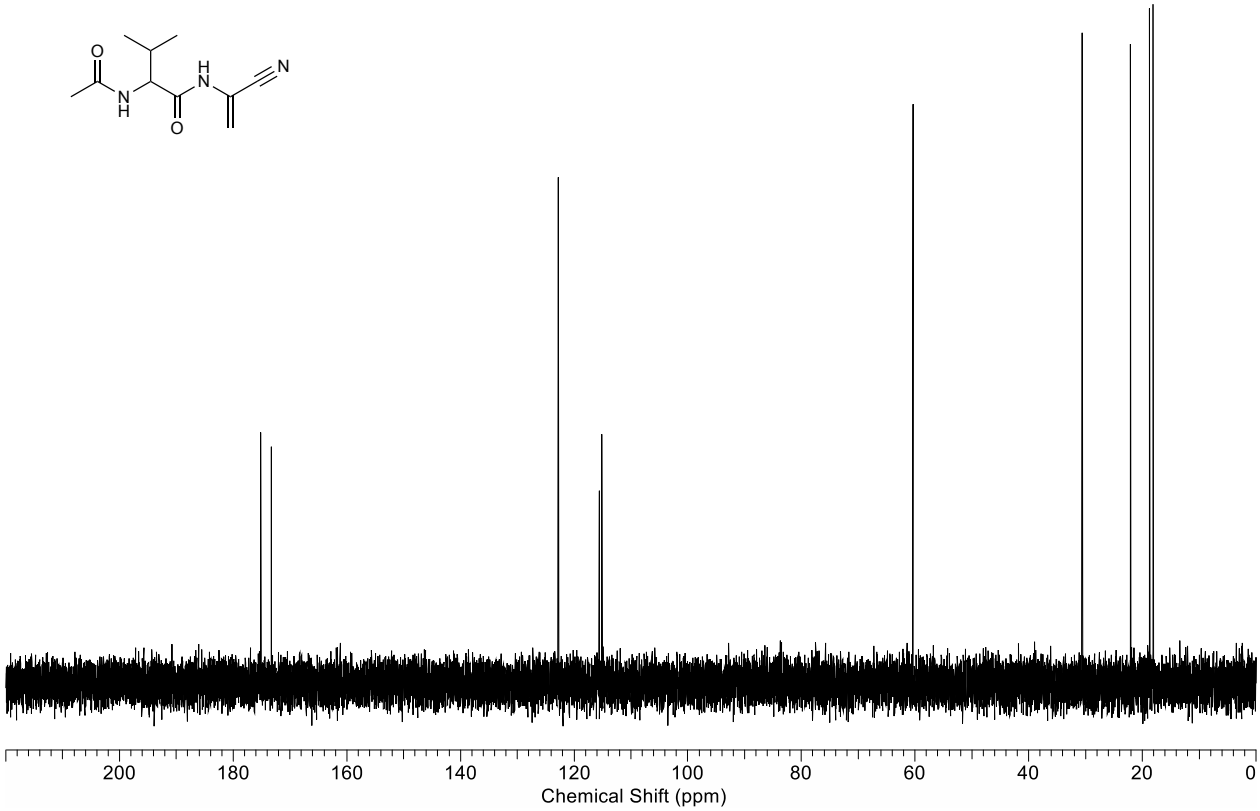
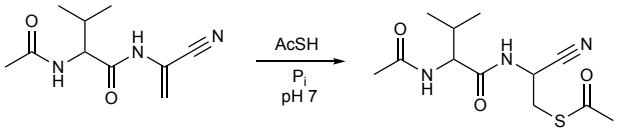


Fig. S62. ^{13}C NMR (176 MHz, D_2O, 0–220 ppm) spectrum to show a *N*-acetyl-DL-valinyldehydroalanine nitrile **Ac-Val-Dha-CN** after flash column chromatography of the crude mixture obtained from the incubation of **Ac-Val-Ser^{Ac}-CN** at pH 8.0 for 4 d.

Addition of thioacetate to N-acetyl-DL-valinyldehydroalanine nitrile in phosphate buffer at pH 7



Ac-Val-Dha-CN **Ac-Val-Cys^{Ac}-CN**

N-Acetyl-DL-valinyldehydroalanine nitrile **Ac-Val-Dha-CN** (24 mg; 0.12 mmol) was dissolved in phosphate buffer (2.28 mL; 500 mM, pH 7). Thioacetic acid **AcSH** (24.6 μL; 0.34 mmol) was added and the resulting solution was incubated at room temperature and periodically analysed by NMR spectroscopy. Conversion to *N*,*S*-diacetyl-DL-valinyl-DL-cysteine nitrile **Ac-Val-Cys^{Ac}-CN** (>95%) was observed after 15 h (Fig. S63). The reaction mixture was concentrated *in vacuo* (T_{bath} = 30 ºC). The residue was triturated with methanol (3 x 10 mL), and the combined methanolic solution was concentrated *in vacuo* and purified by flash column chromatography (SiO_2; EtOAc (100%)) to give *N,S*-diacetyl-DL-valinyl-DL-cysteine nitrile **Ac-Val-Cys^{Ac}-CN** (16 mg, 38%) as a colourless film (Fig. S64). ^{1}H NMR (700 MHz, D_2O) δ 5.01 - 4.96 (m, 2H, Cys-(C2)–H × 2), 4.04 (d, *J* = 6.7 Hz, 1H, Val-(C2)–H), 4.02 (d, *J* = 7.2 Hz, 1H, Val-(C2)–H), 3.47 - 3.33 (m, 4H, Cys-(C3)–H_2 × 2), 0.95 - 0.90 (m, 12H, Val-(C4)–H_3 × 2, Val-(C4′)–H_3 × 2), 2.39 (s, 6H, SCOCH_3 × 2), 2.08 - 2.04 (m, 2H, Val-(C3)–H × 2), 2.04 (s, 3H, COCH_3), 2.03 (s, 3H, COCH_3). ^{13}C NMR (176 MHz, D_2O) (2 diastereoisomers, 1:1) δ 199.5 (S**C**OCH_3), 199.4 (S**C**OCH_3), 175.0 (**C**OCH_3), 175.0 (**C**OCH_3), 174.3 (Val-(C1)), 174.3 (Val-(C1)), 117.9 (Cys-(C1)), 117.9 (Cys-(C1)), 60.2 (Val-(C2)), 60.2 (Val-(C2)), 41.2 (Cys-(C2)), 41.1 (Cys-(C2)), 30.6 (Cys-(C3)), 30.6 (Cys-(C3)), 30.5 (Val-(C3)), 30.5 (Val-(C3)), 30.4 (SCO**C**H_3 × 2), 22.1 (CO**C**H_3 × 2), 18.8 (Val-(C4)), 18.8 (Val-(C4)), 18.1 (Val-(C4′)), 18.0 (Val-(C4′)). HRMS-ESI $[M+H^+]^+$ calculated for $C_{12}H_{20}N_3O_3S^+$, 286.1219; found 286.1220. IR (cm^{-1}): 3277, 2965, 2936, 2876, 2418, 1632, 1539.

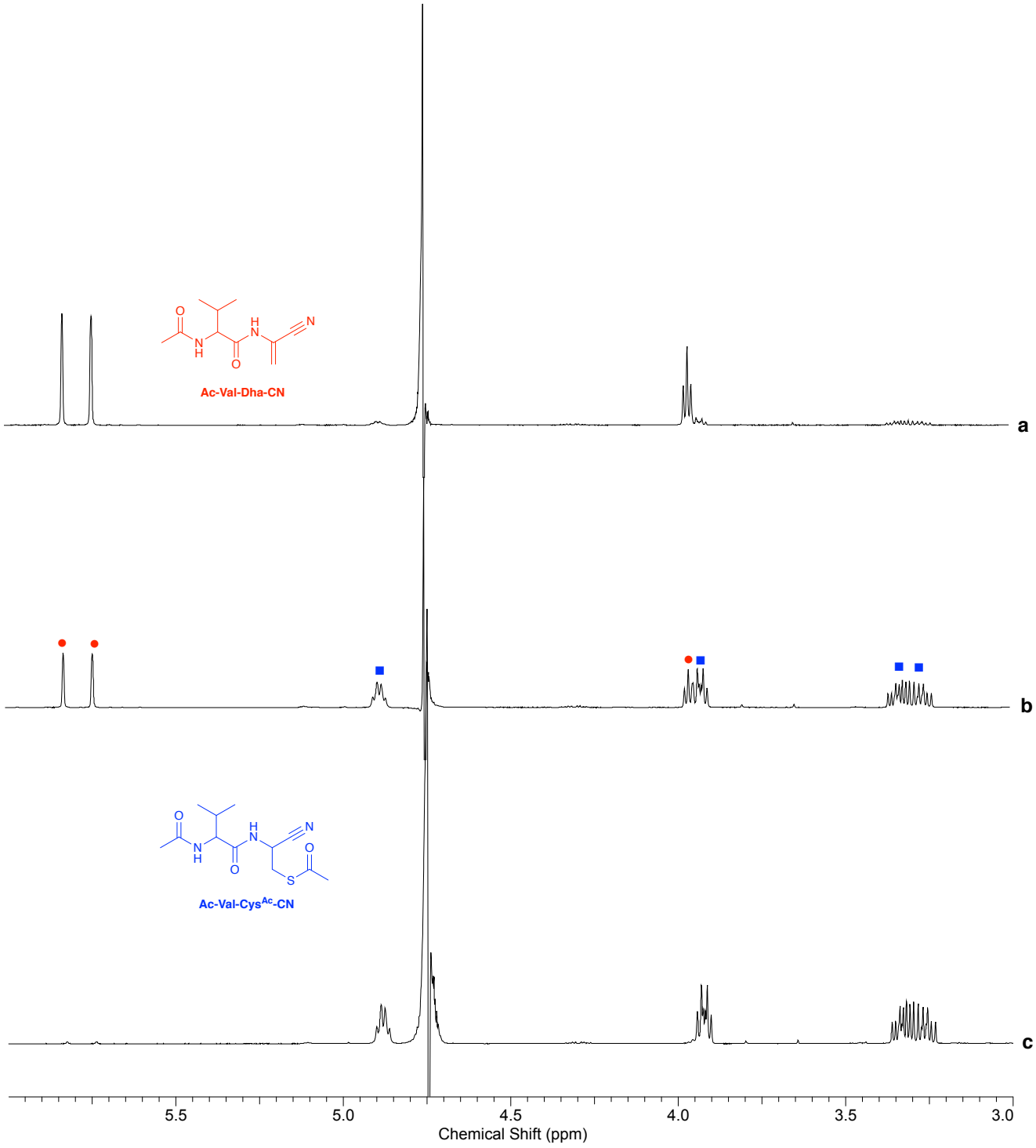


Fig. S63. Stacked ^{1}H NMR (600 MHz; H_2O, noesygppr1d) spectra to show the formation of *N,S*-diacetyl-DL-valinyl-DL-cysteine nitrile (**Ac-Val-Cys^{Ac}-CN**) by reaction of *N*-acetyl-DL-valinyldehydroalanine nitrile (**Ac-Val-Dha-CN**; 50 mM) with thioacetic acid (**AcSH**; 150 mM) in phosphate buffer (500 mM) at pH 7 and room temperature. **a** = 10 min; **b** = 3 h; **c** = 12 h.

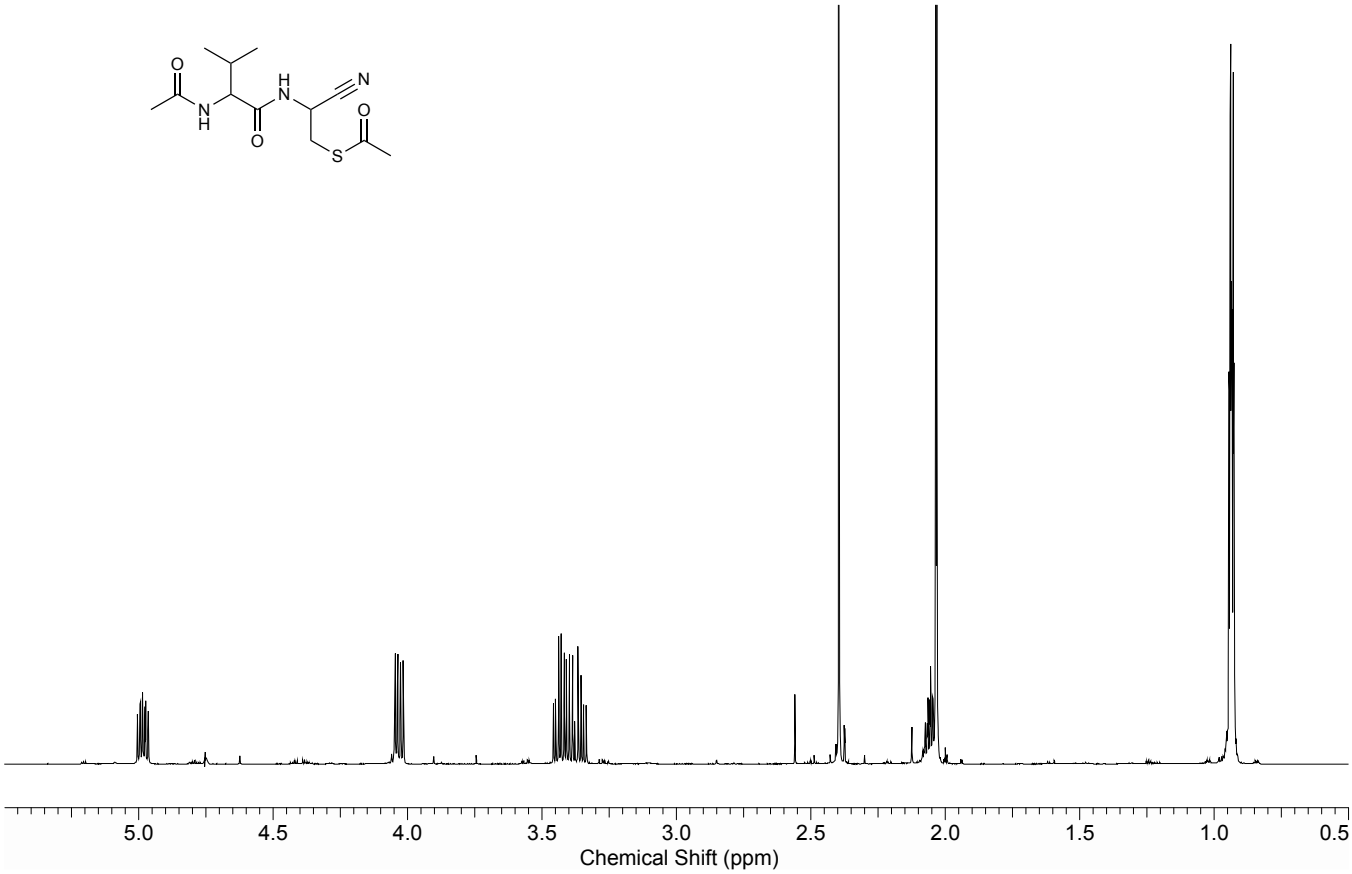


Fig. S64. ^{1}H NMR (700 MHz, D_2O, 0.5–55 ppm, noesygppr1d) spectrum to show *N,S*-acetyl-DL-valinyl-DL-cysteine nitrile (**Ac-Val-Cys^{Ac}-CN**).

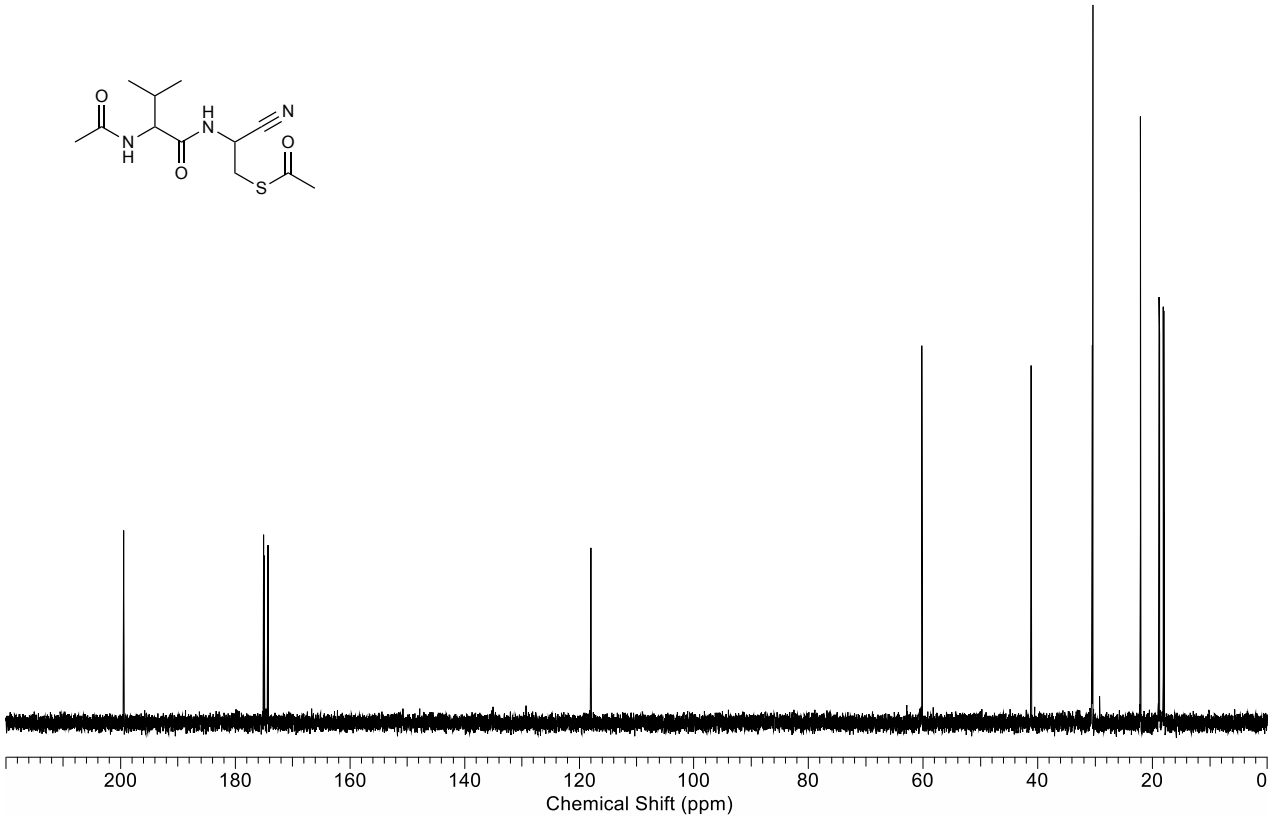


Fig. S65. ^{13}C NMR (176 MHz, D_2O, 0–220 ppm) spectrum to show *N,S*-acetyl-DL-valinyl-DL-cysteine nitrile (**Ac-Val-Cys^{Ac}-CN**).

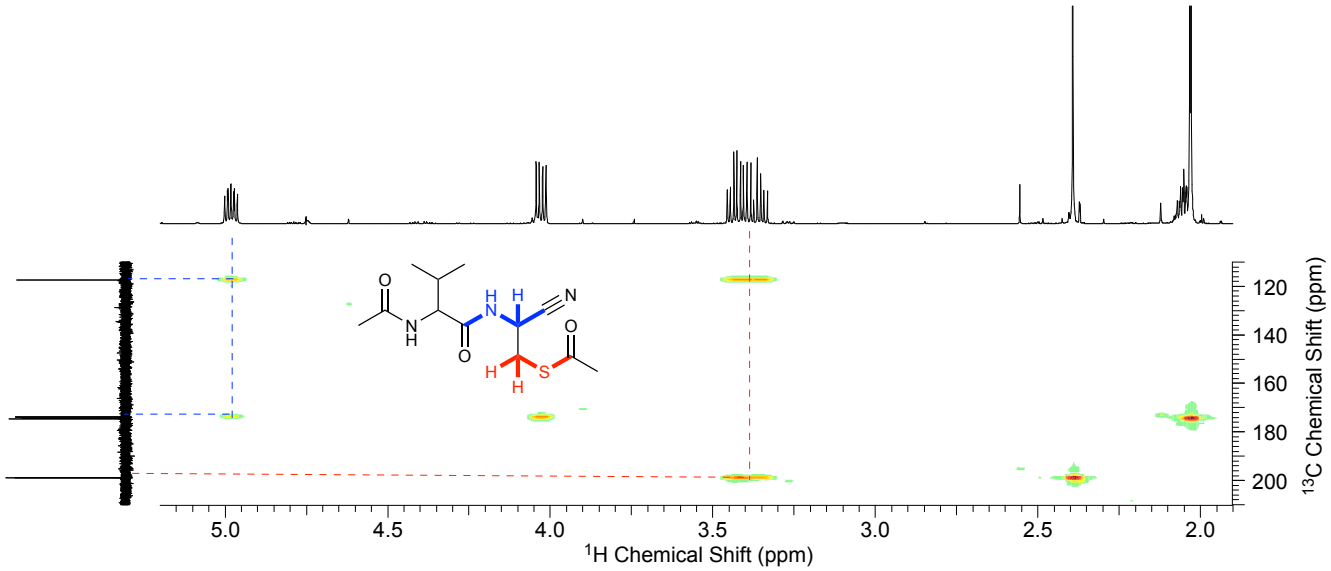
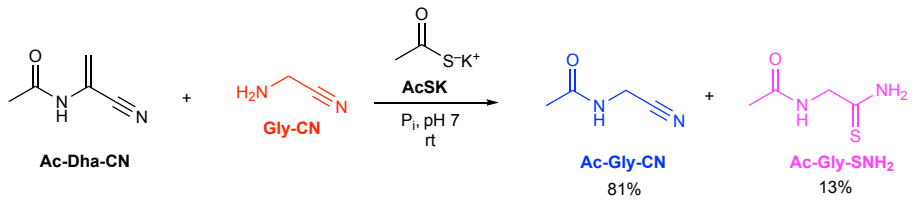


Fig. S66. ^{1}H–^{13}C HMBC (^{1}H–700 MHz [1.9–5.2 ppm], ^{13}C–176 MHz [110–210 ppm], D_2O) spectrum showing diagnostic $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling of Cys-(C2)–H of **Ac-Val-Cys^{Ac}-CN** at 4.96-5.01 ppm with CN resonances at 117.9 ppm and the Val-(C1) resonances at 174.3 ppm (shown in blue). Diagnostic $^{3}J_{CH}$ coupling of Cys-(C3)–H_2 at 3.33-3.47 pm with the thioester S**C**OCH_3 at 199.4 and 199.5 ppm are highlighted in red.

One-pot cysteine thioester-mediated aminonitrile acetylation



N-Acetyldehydroalanine nitrile **Ac-Dha-CN** (60 mM), potassium thioacetate (**AcSK**; 120 mM) and glycine nitrile (**Gly-CN**; 60 mM) were dissolved in phosphate buffer (500 mM, pH 7). The acetylation of Gly-CN was periodically monitored by acquisition of NMR spectra. Near-quantitative acetylation of glycine nitrile **Gly-CN** was observed after 3 d, to afford **Ac-Gly-CN** (81%) and **Ac-GlySNH$_2$** (13%) (Fig. S67).

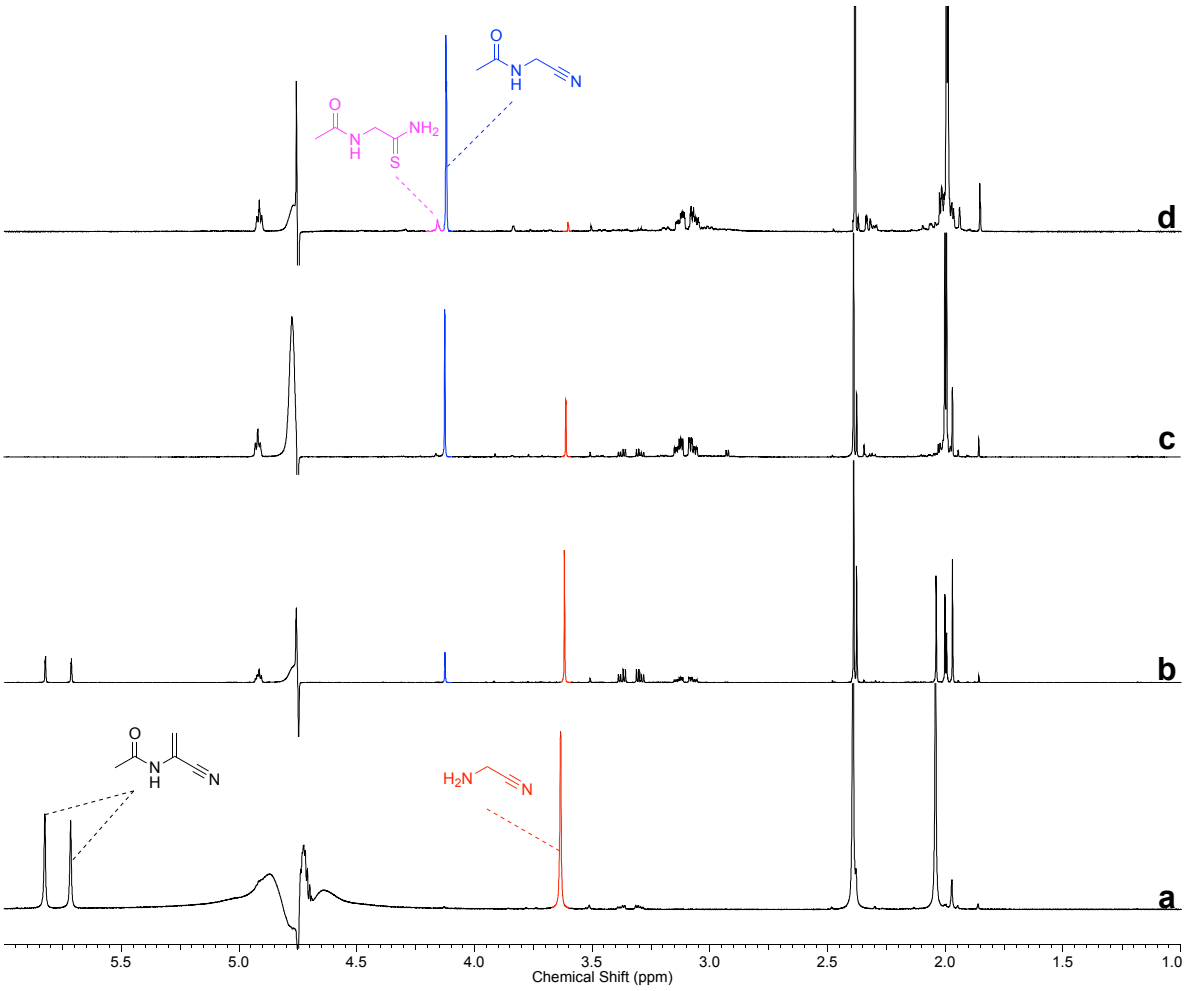
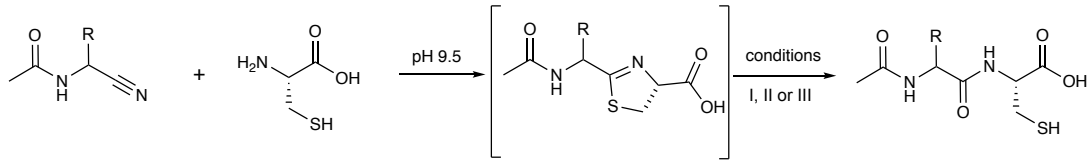


Fig. S67. ^{1}H NMR spectra (700 MHz, H_2O, noesygppr1d, 1.00–6.00 ppm) to show the reaction of **Ac-Dha-CN** (60 mM) with potassium thioacetate (**AcSK**; 120 mM) and **Gly-CN** (60 mM) in phosphate buffer (250 mM, pH 7.0) at room temperature after: **a**) 1 h; **b**) 4 h; **c**) 1 d; **d**) 3 d.

Synthesis of α-amidocysteines by coupling of α-amidonitriles with L-cysteine



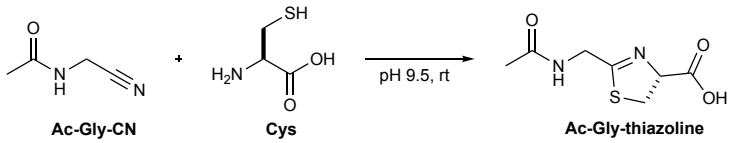
Ac-AA-CN **Cys** **Ac-AA-thiazoline** **Ac-AA-Cys-OH**

General procedure

α-Amidonitrile (**Ac-AA-CN**; 200 mM) and L-cysteine (**Cys**; 1–2 equiv.) were dissolved in water and the solution was adjusted to pH 9.5 with HCl/NaOH. The solution was stirred at room temperature for 1 – 4 h, until the complete consumption of **Ac-AA-CN** was observed to give a thiazoline intermediate **Ac-AA-thiazoline**. The solution was then adjusted to either (**I**) pH 1.5 and incubated at room temperature, (**II**) pH 7 and incubated at room temperature, (**III**) pH 7 and incubated at 60 °C. The reactions were incubated until complete consumption of **Ac-AA-thiazoline** was observed by NMR spectroscopy. The formation of **Ac-AA-Cys-OH** confirmed by NMR spectroscopy and high-resolution mass spectrometry.

N-Acetylglycyl-L-cysteine synthesis via thiazoline intermediate

Thiazoline formation from N-acetylglycine nitrile with L-cysteine



Ac-Gly-CN **Cys** **Ac-Gly-thiazoline**

N-Acetyglycine nitrile (**Ac-Gly-CN**; 200 mM) and L-cysteine (**Cys**; 200 mM) were dissolved in H_2O/D_2O (9:1; 1 mL) and the solution was adjusted to pH 9.5 with HCl/NaOH. The solution was then stirred at room temperature for 1 h. Quantitative conversion to **Ac-Gly-thiazoline** was observed (Fig. S68 and Fig. S69). ^{1}H NMR (700 MHz ,H_2O:D_2O, 9:1, noesygppr1d) δ 4.95 (dddd, *J* = 1.6, 1.6, 8.2, 9.8 Hz, 1H, (C4)–H), 4.22 (dd, *J* = 1.6, 16.9 Hz, 1H, Gly-(C2)–H), 4.19 (dd, *J* = 1.6, 16.9 Hz, 1H, Gly-(C2)–H′), 3.63 (dd, *J* = 9.8, 11.2 Hz, 1H, (C5)–H), 3.40 (dd, *J* = 8.2, 11.2 Hz, 1H, (C5)–H′). ^{13}C NMR (176 MHz, H_2O:D_2O, 9:1) δ 179.0 (C2), 175.2 (CO_2H), 174.6 (**C**OCH_3), 80.2 (C4), 42.1 (Gly-(C2)), 36.9 (C5), 22.4 (CO**C**H_3).

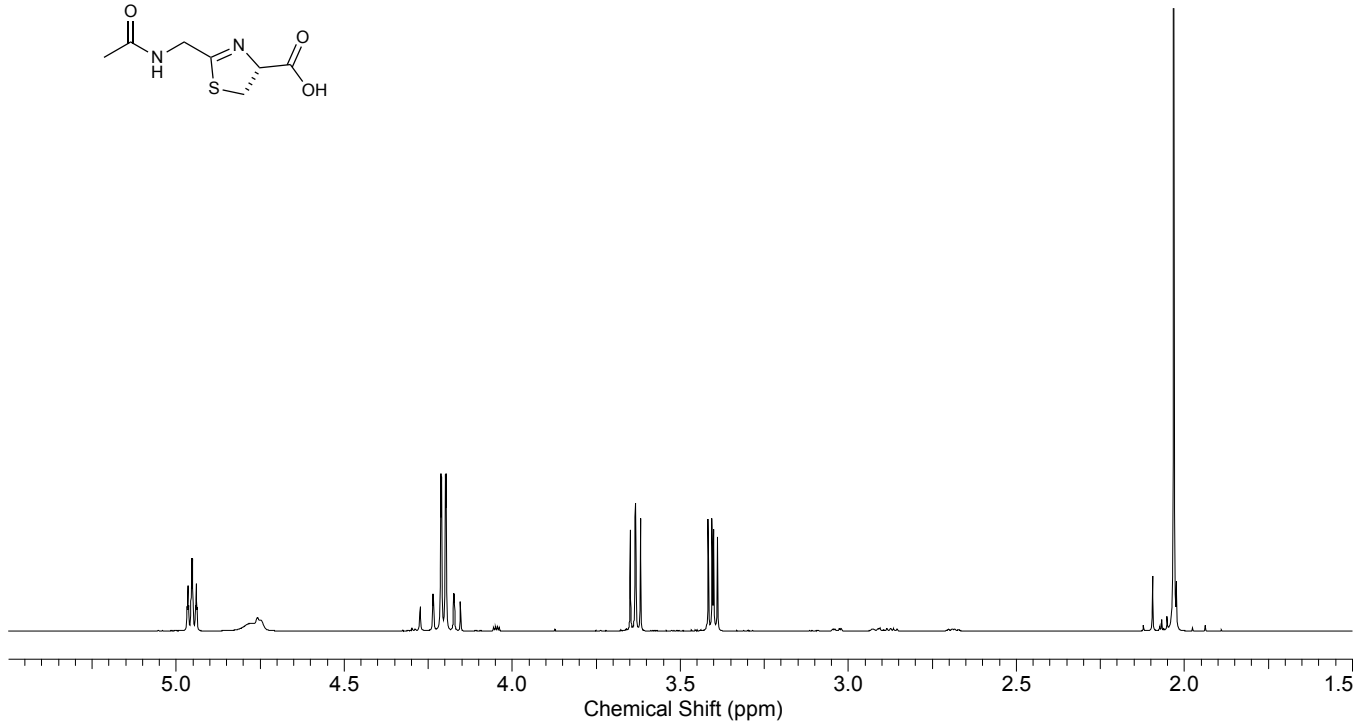


Fig. S68. ^{1}H NMR (700 MHz, H_2O/D_2O, 1.5–5.5 ppm, noesygppr1d) spectrum to show the formation of **Ac-Gly-thiazoline** from the reaction of *N*-acetylglycine nitrile (**Ac-Gly-CN**; 200 mM) with L-cysteine (**Cys**; 200 mM) at pH 9.5 and room temperature.

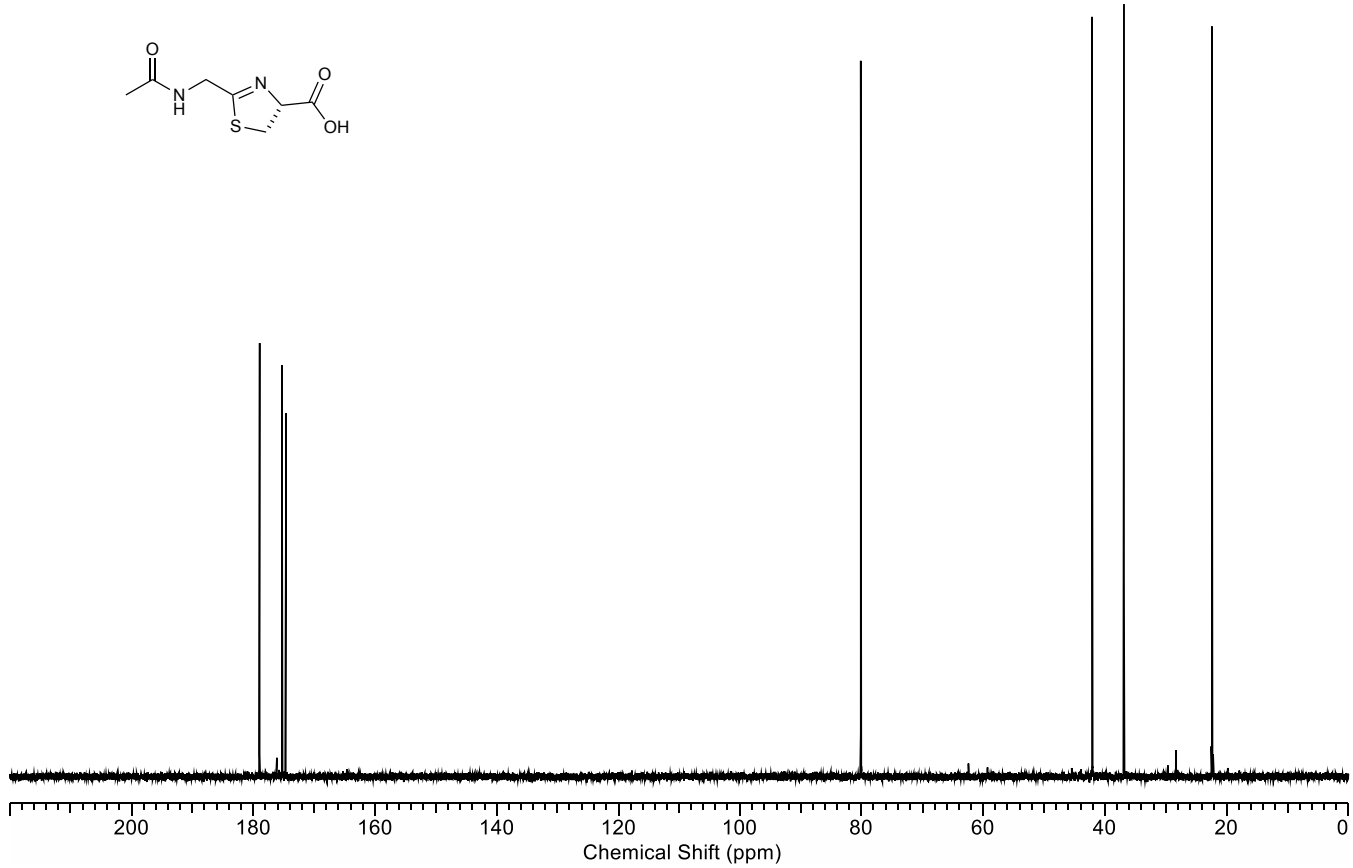
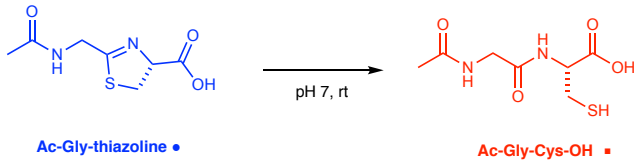


Fig. S69. ^{13}C NMR (176 MHz, H_2O/D_2O (9:1), 0–220 ppm) spectrum to show the formation of **Ac-Gly-thiazoline** from the reaction of *N*-acetylglycine nitrile (**Ac-Gly-CN**; 200 mM) with L-cysteine (**Cys**; 200 mM) at pH 9.5 and room temperature.

Time course of ***Ac-Gly-thiazoline*** *hydrolysis to N-acetylglycyl-L-cysteine* ***Ac-Gly-Cys-OH*** *at pH 7 and room temperature*



Ac-Gly-thiazoline (200 mM) in H_2O/D_2O (9:1) was adjusted to pH 7 with HCl. The reaction was monitored by ^1H NMR spectroscopy (Fig. S70) at room temperature. The hydrolysis of **Ac-Gly-thiazoline** to *N*-acetylglycyl-L-cysteine **Ac-Gly-Cys-OH** was observed over 5 d (**Ac-Gly-thiazoline** (8%); **Ac-Gly-Cys-OH** (78%), Fig. S70d). δ 4.40 (dd, *J* = 4.7, 6.0 Hz, 1H, Cys-(C2)–H), 3.98 – 3.0 (m, 2H, Gly-(C2)–H_2), 2.94 (ABX, *J* = 4.7, 13.9 Hz, 1H, Cys-(C3)–H), 2.91 (ABX, *J* = 6.0, 13.9 Hz, 1H, Cys-(C3)–H′), 2.06 (s, 3H, $COCH_3$).

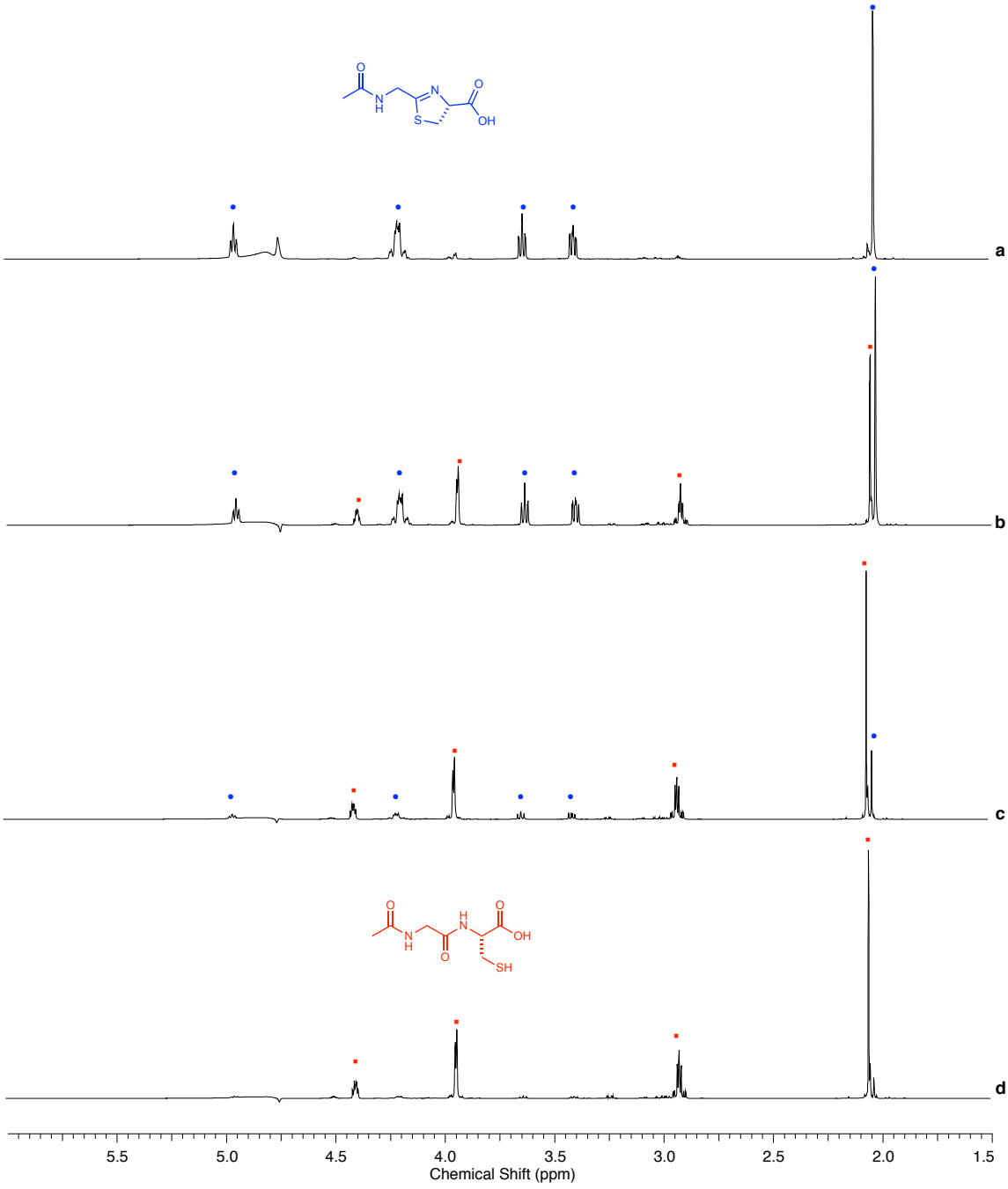
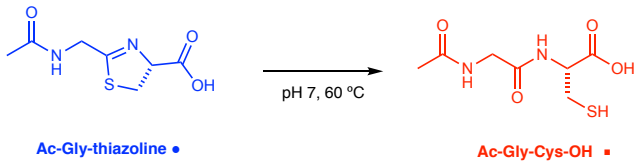


Fig. S70. ^1H NMR (700 MHz, $H_2O:D_2O$, 9:1, noessygppr1d, 1.5-6.0 ppm) spectra to show the hydrolysis of **Ac-Gly-thiazoline** (200 mM) to *N*-acetylglycyl-L-cysteine **Ac-Gly-Cys-OH** after **a.** 3h; **b.** 1 d; **c.** 3 d; **d**. 5 d.

*Time course of **Ac-Gly-thiazoline** hydrolysis to N-acetylglycyl-L-cysteine **Ac-Gly-Cys-OH** at pH 7 and 60 ℃*



Ac-Gly-thiazoline ● Ac-Gly-Cys-OH ▪

Ac-Gly-thiazoline (200 mM) in H_2O/D_2O (9:1) was adjusted to pH 7 with HCl. The reaction was heated at 60 °C for 2 h before rapidly cooling the reaction to 0 ºC. The hydrolysis of **Ac-Gly-thiazoline** to *N*-acetylglycyl-L-cysteine **Ac-Gly-Cys-OH** (90%) was observed by ^{1}H and ^{13}C NMR spectroscopy (Fig. S71 and Fig. S72). ^{1}H NMR (700 MHz, H_2O/D_2O (9:1), noesygppr1d) δ 4.40 (dd, *J* = 4.8, 5.8 Hz, 1H, Cys-(C2)–H), 3.96 – 3.92 (m, 2H, Gly-(C2)–H_2), 2.94 (ABX, *J* = 4.8, 13.9 Hz, 1H, Cys-(C3)–H), 2.91 (ABX, *J* = 5.8, 13.9 Hz, 1H, Cys-(C3)–H′), 2.06 (s, 3H, $COCH_3$). ^{13}C NMR (176MHz, H_2O/D_2O, 9:1) δ 176.7 (Cys-(C1)), 175.6 (**C**OCH_3), 171.6 (Gly-(C1)), 57.1 (Cys-(C2)), 43.4 (Gly-(C2)), 27.0 (Cys-(C3)), 22.5 (CO**C**H_3). HRMS-ESI $[M+H]^+$ calculated for $C_7H_{13}N_2O_4S$, 221.0593; observed 221.0596.

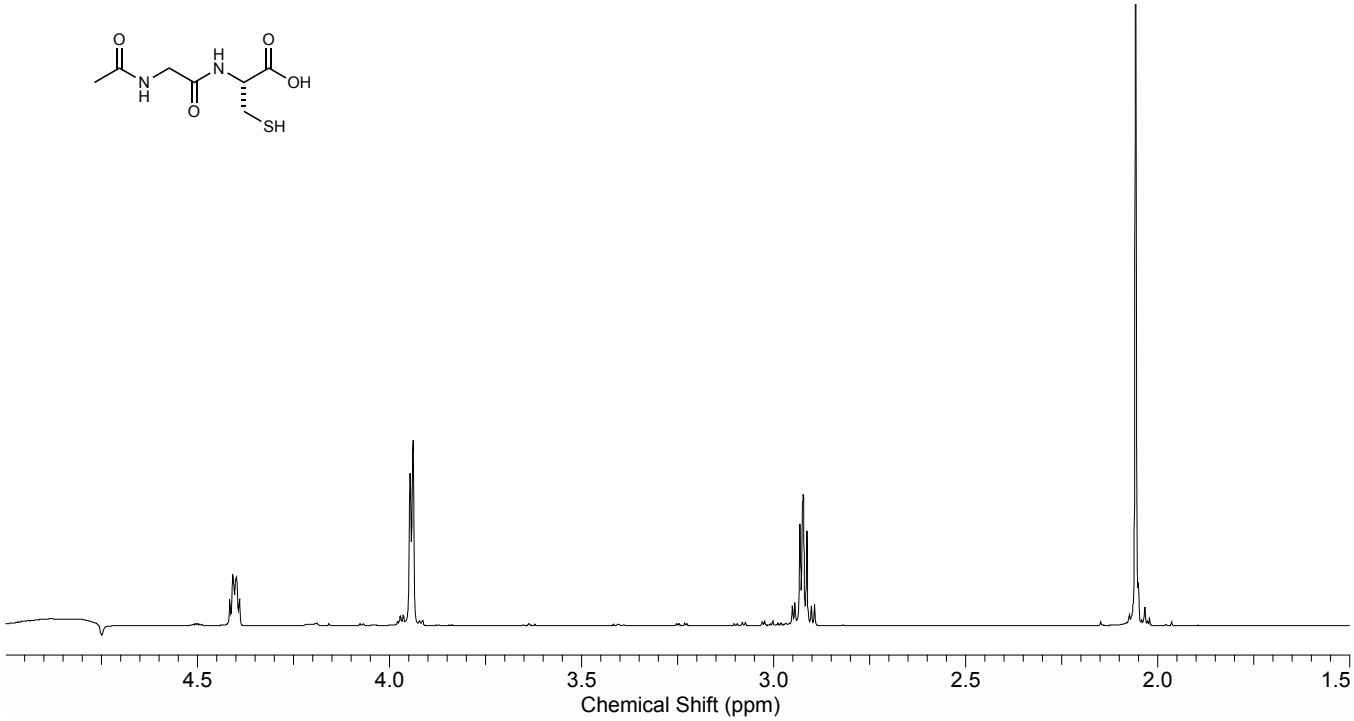


Fig. S71. ^{1}H NMR (700 MHz, H_2O/D_2O, 9:1, 1.5–5.0 ppm, noesygppr1d) spectrum to show the conversion of **Ac-Gly-thiazoline** to **Ac-Gly-Cys-OH** after 2 h at pH 7 and 60 °C.

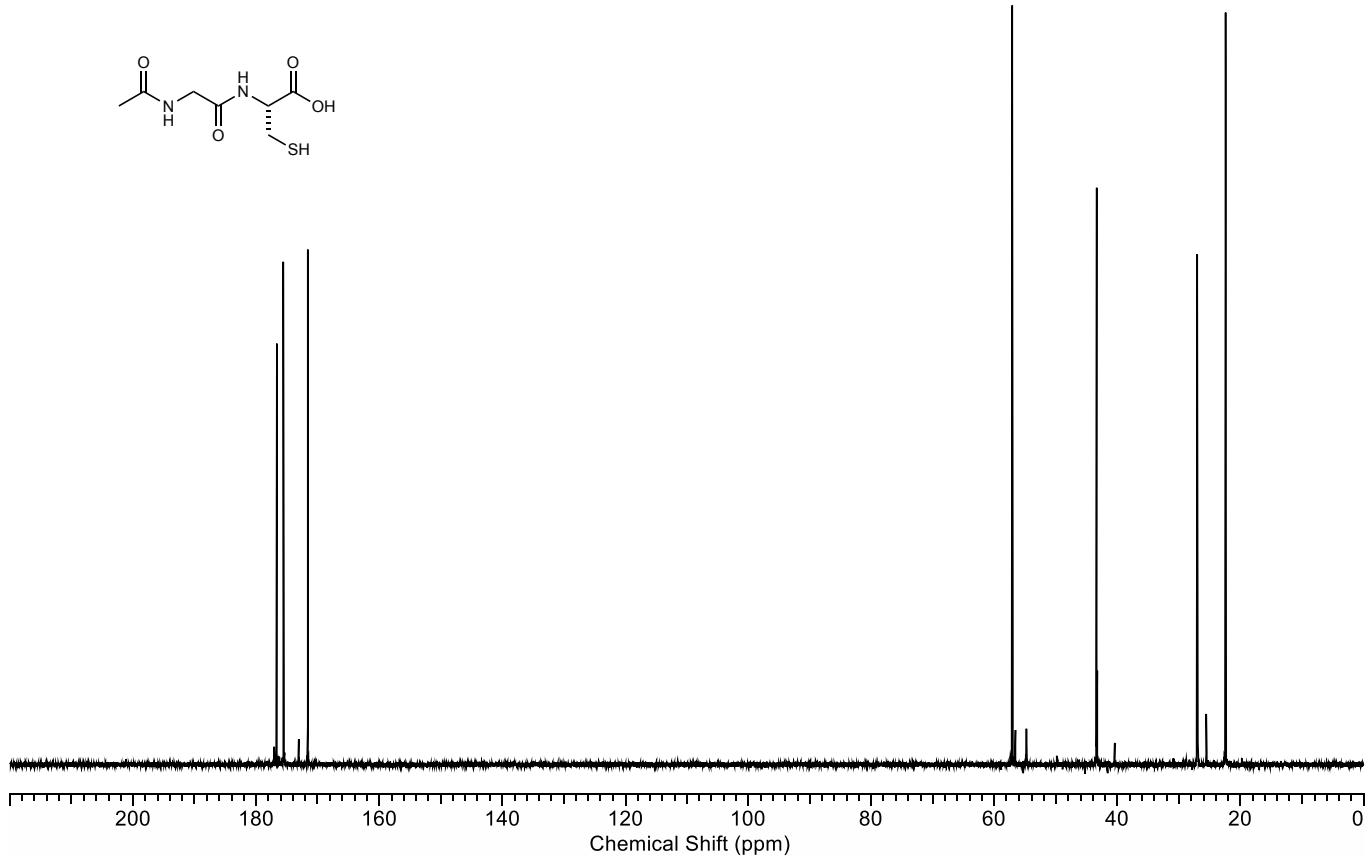
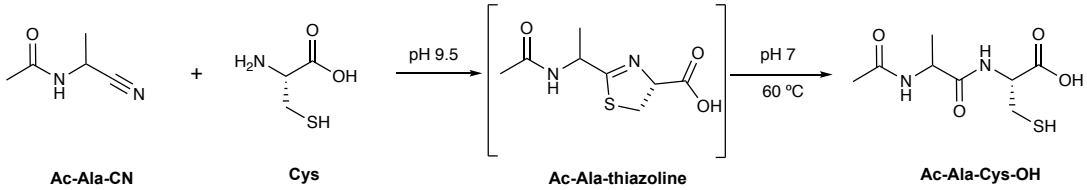


Fig. S72. ^{13}C NMR (176 MHz, H_2O/D_2O (9:1), 1.5–5.0 ppm, noesygppr1d) spectrum to show the conversion of **Ac-Gly-thiazoline** to **Ac-Gly-Cys-OH** after 2 h at pH 7 and 60 °C.

N-Acetyl-DL-alanyl-L-cysteine



N-Acetyl-DL-alanine nitrile (**Ac-Ala-CN**; 22.4 mg, 0.20 mmol) and L-cysteine (**Cys**; 24.2 mg, 0.20 mmol) were dissolved in H_2O/D_2O (9:1; 1 mL). The solution was adjusted to pH 9.5 with HCl/NaOH and incubated at room temperature for 2 h to give **Ac-Ala-thiazoline**. The solution was then adjusted to pH 7 with 4M HCl and then heated at 60 °C for 24 h. The resulting diastereoisomeric mixture of **Ac-Ala-Cys-OH** was used without further purification.

*Data for **Ac-Ala-thiazoline***

^{1}H NMR (600 MHz, H_2O/D_2O 9:1, noesygppr1d, 2 diastereoisomers (50:50; a:b)): δ 4.90 - 4.82 (m, 2H, (C4)–H_a, (C4)–H_b), 4.67 - 4.58 (m, 2H, Ala-(C2)–H_a, Ala-(C2)–H_b), 3.58 - 3.47 (m, 4H, (C5)–H_a, (C5)–H_b), 3.32 - 3.25 (m, 4H, (C5)–H_a', (C5)–H_b'), 1.92 (s, 3H, $COCH_{3a}$), 1.92 (s, 3H, $COCH_{3b}$), 1.34 (d, *J* = 7.3 Hz, 3H, Ala-(C3)–H_{3a}), 1.33 (d, *J* = 7.3 Hz, 3H, Ala-(C3)–H_{3b}). ^{13}C NMR (176 MHz, H_2O/D_2O 9:1, 2 diastereoisomers (50:50; a:b)):) δ 179.0 ($C2_a$), 179.0 ($C2_a$), 178.9 (CO_2H_a), 178.8 (CO_2H_a), 174.6 (**C**OCH_{3a}), 174.4 (**C**OCH_{3b}), 80.6 ($C4_a$), 80.5 ($C4_b$), 48.9 (Ala-($C2_a$)), 48.8 (Ala-($C2_a$)), 36.8 ($C5_a$), 36.5 ($C5_b$), 22.4 (CO**C**H_{3a}), 22.4 (CO**C**H_{3b}), 18.9 (Ala-($C3_a$)), 18.8 (Ala-($C3_b$)).

*Data for **Ac-Ala-Cys-OH***

^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d, 2 diastereoisomers (50:50)): δ 4.39–4.36 (2H, m, Cys-(C2)–H), 4.29–4.25 (2H, m, Ala-(C2)–H), 2.91–2.85 (4H, m, Cys-(C3)–H_2), 1.99 (3H, s, $COCH_3$), 1.97 (3H, s, $COCH_3$), 1.35 (3H, d, *J* = 7.3 Hz, Ala-(C3)–H_3), 1.33 (3H, d, *J* = 7.2 Hz, Ala-(C3)–H_3). ^{13}C NMR (176 MHz; H_2O/D_2O 9:1, 2 diastereoisomers (50:50)): δ 176.6 (Cys-(C1)), 176.6 (Cys-(C1)), 175.2 (Ala-(C1))), 175.2 (Ala-(C1)), 174.8 (**C**OCH_3), 174.7 (**C**OCH_3), 57.2 (Cys-(C2)), 56.9 (Cys-(C2)), 50.7 (Ala-(C2)), 50.5 (Ala-(C2)), 27.1 (Cys-(C3)), 27.0 (Cys-(C3)), 22.4 (CO**C**H_3), 22.3 (CO**C**H_3), 17.5 (Ala-(C3)), 17.2 (Ala-(C3)). HRMS-ESI $[M+H]^+$ calculated for formula $C_8H_{15}N_2O_4S^+$, 235.0752; found 235.0753. IR (cm^{-1}): 3323, 3138, 3044, 2963, 1736, 1644, 1576, 1537.

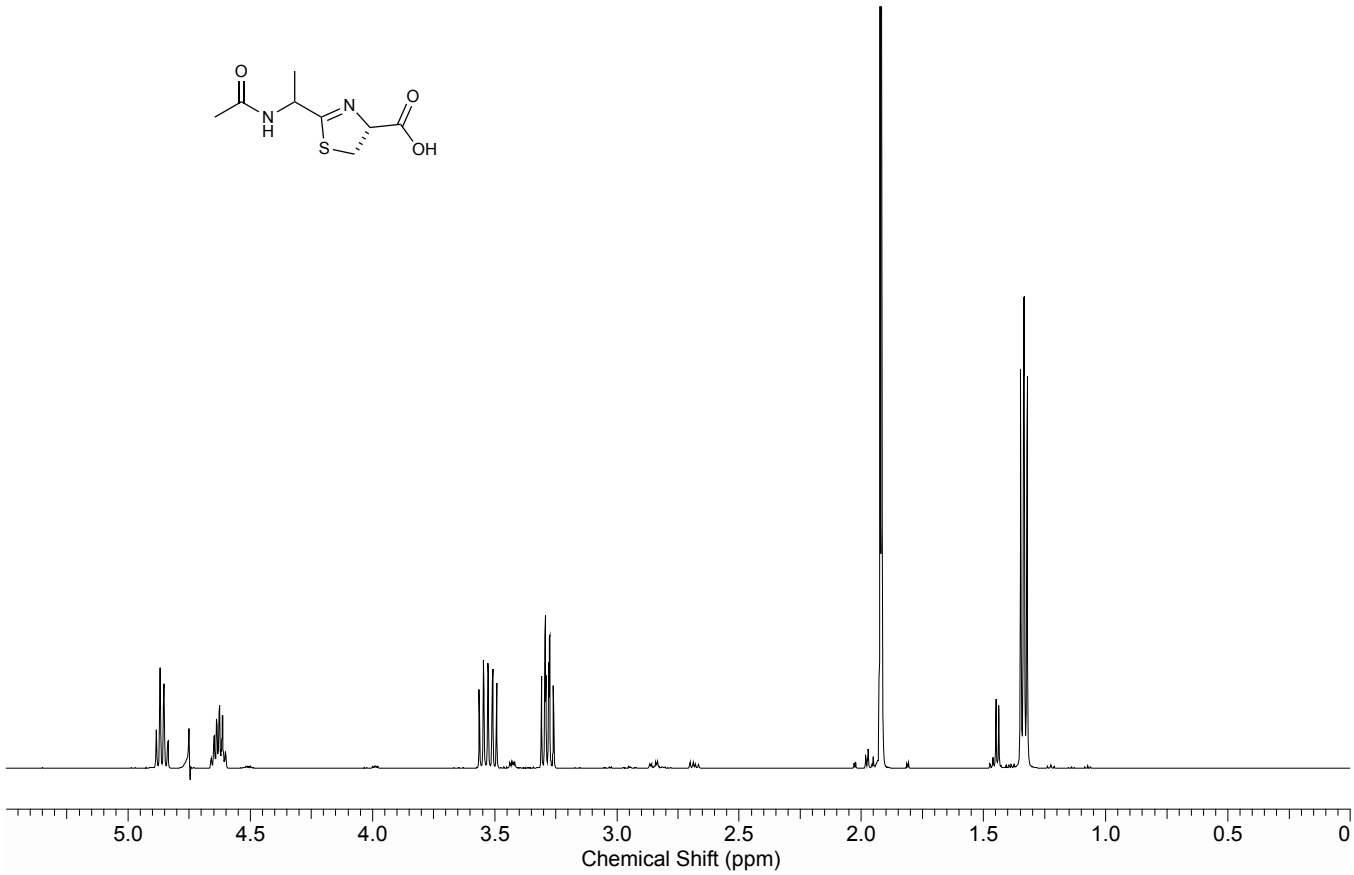


Fig. S73. ^{1}H NMR (600 MHz, H_2O/D_2O 9:1, noesygppr1d, 0.00–5.50 ppm) spectrum of **Ac-Ala-thiazoline**.

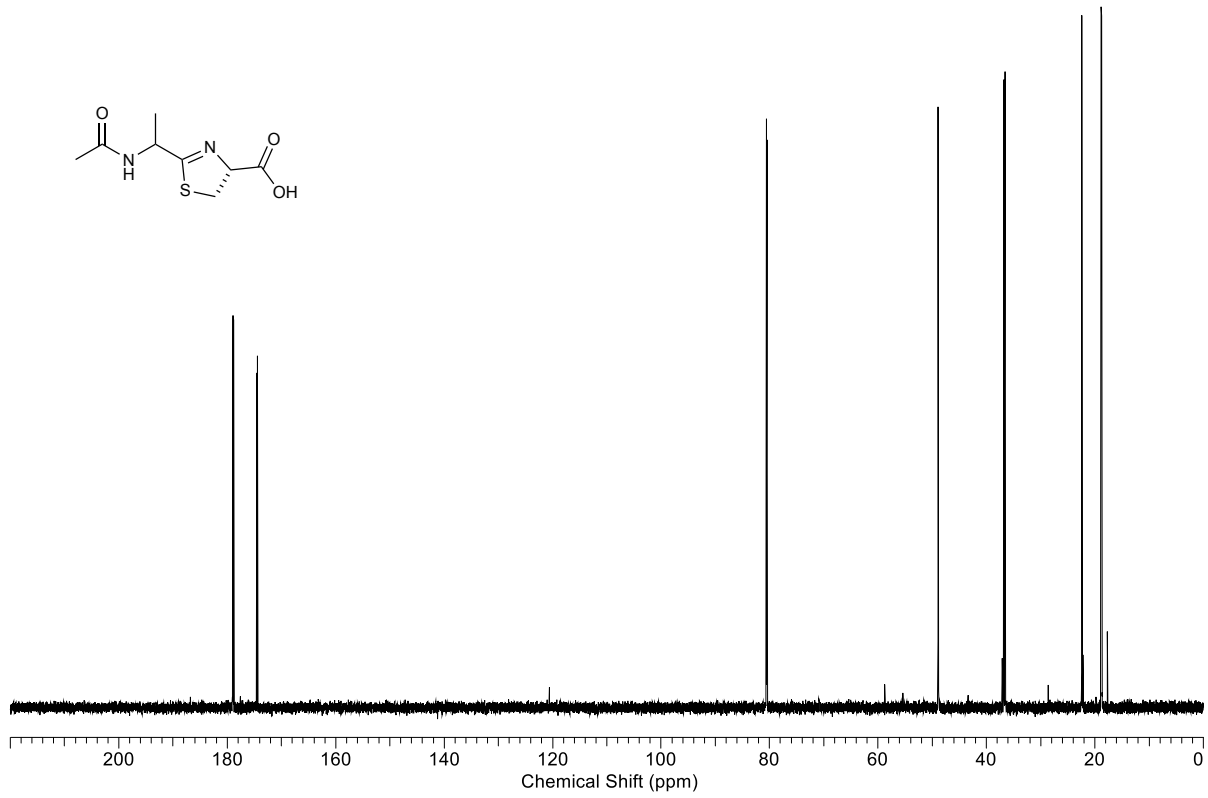


Fig. S74. ^{13}C NMR (176 MHz, H_2O/D_2O 9:1, 0–220 ppm) spectrum of **Ac-Ala-thiazoline**.

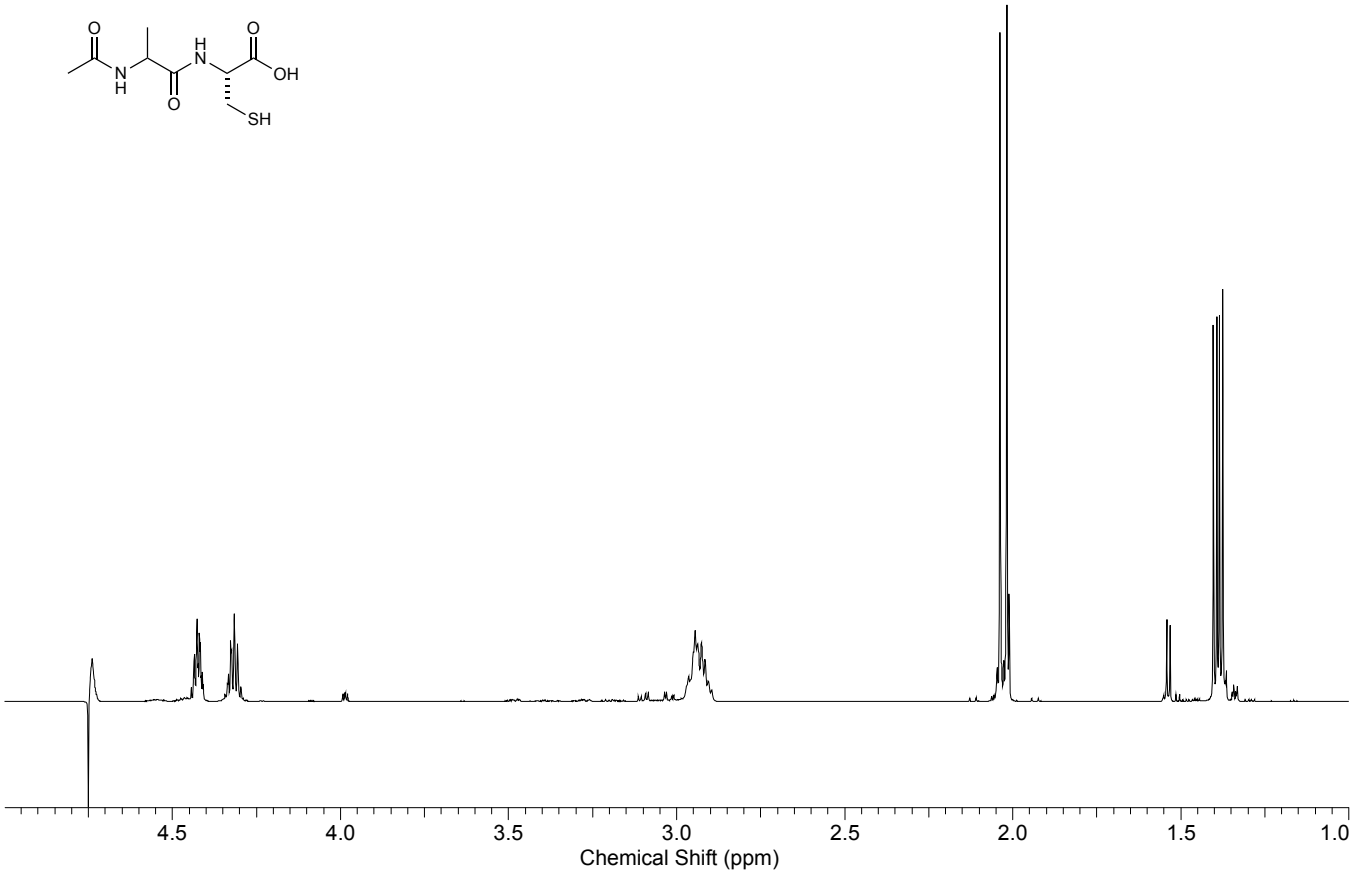


Fig. S75. ^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d, 1.00–5.50 ppm) spectrum of **Ac-Ala-Cys-OH**.

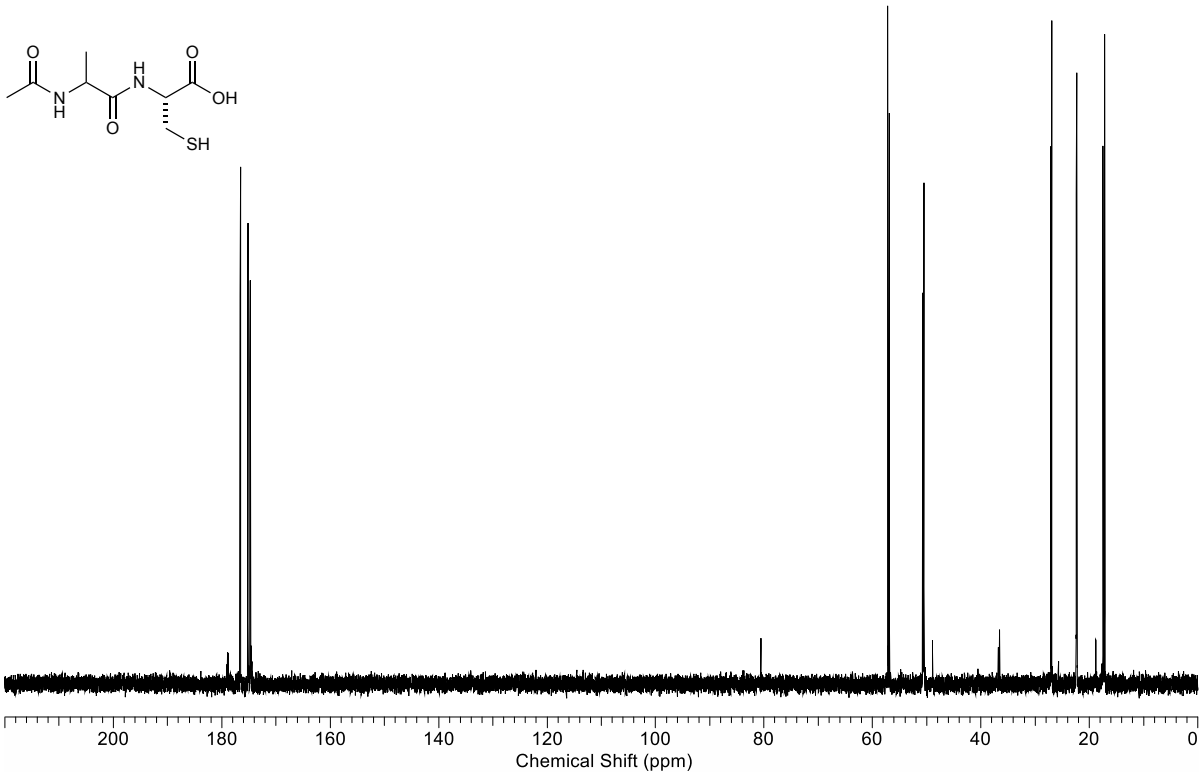
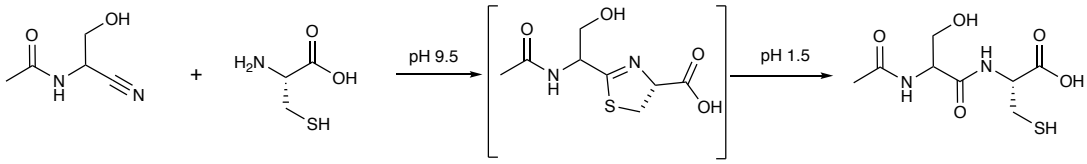


Fig. S76. ^{13}C NMR (176 MHz, H_2O/D_2O 9:1, 0–220 ppm) spectrum of **Ac-Ala-Cys-OH**.

N-Acetyl-DL-serinyl-L-cysteine



Ac-Ser-CN **Cys** **Ac-Ser-thiazoline** **Ac-Ser-Cys-OH**

N-Acetyl-DL-serine nitrile (**Ac-Ser-CN**; 204 mg, 1.59 mmol) and L-cysteine (**Cys**; 193 mg, 1.59 mmol) in H_2O/D_2O (8 mL) was adjusted to pH 9.5 and stirred at room temperature for 1 h to give **Ac-Ser-thiazoline**. The solution was decreased to pH 1.5 and then stirred at room temperature for 12 h. After this time the reaction mixture was concentrated *in vacuo* and the residue purified by flash column chromatography (SiO_2; isopropanol/water 9:1) to yield 250 mg of a diastereomeric mixture of **Ac-Ser-Cys-OH** as a colourless solid.

*Data for **Ac-Ser-thiazoline***

^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d, 2 diastereoisomers (50:50; a:b)): δ 4.97 (ddd, J = 1.3, 8.5, 9.6 Hz, 1H, (C4)–H_a), 4.93 (ddd, J = 1.6, 8.8, 9.9 Hz, 1H, (C4)–H_b), 4.80 - 4.76 (obs., 2H, Ser-(C2)–H_a; Ser-(C2)–H_b), 3.90 - 3.82 (m, 4H, Ser-(C3)–H_{2a}; Ser-(C3)–H_{2b}), 3.64 - 3.56 (m, 2H, (C5)–H_a, (C5)–H_b), 3.39 - 3.34 (m, 2H, (C5)–H_a', (C5)–H_b'), 2.03 (2 $\times$ s, 6H, $COCH_{3a}$, $COCH_{3b}$). ^{13}C NMR (176 MHz, H_2O/D_2O 9:1, 2 diastereoisomers (50:50; a:b)) (partial assignment): δ 178.9 (CO_2H_a), 178.8 (CO_2H_b), 175.6, 175.1, 175.1, 175.0, 80.7 ($C4_a$), 80.6 ($C4_b$), 62.7 (Ser-($C2_a$), 62.7 (Ser-($C2_b$), 54.7 $\times$ 2 ((Ser-($C3_a$); (Ser-($C3_b$)), 36.8 ($C5_a$), 36.6 ($C5_b$), 22.5 (CO**C**H_{3a}), 22.4 (CO**C**H_{3b}).

*Data for **Ac-Ser-Cys-OH***

^{1}H NMR (700 MHz, D_2O, noesygppr1d): δ 4.64 - 4.59 (m, 2H, Cys-(C2)–H_a; Cys-(C2)–H_b), 4.47 (app. t, J = 5.5 Hz, 2H, Ser-(C2)–H_a; Ser-(C2)–H_b), 3.87 (dd, J = 4.9, 11.7 Hz, 2H, Ser-(C3)–H_a; Ser-(C3)–H_b), 3.84 (dd, J = 6.1, 11.7 Hz, 2H, Ser-(C3)–H_a'; Ser-(C3)–H_b'), 3.02 - 2.93 (m, 4H, Cys-(C3)–H_{2a}; Cys-(C3)–H_{2b}), 2.06 (s, 3H $COCH_{3a}$), 2.06 (s, 3H, $COCH_{3b}$). ^{13}C NMR (176 MHz, D_2O): δ 174.50 (CO**C**H_{3a}), 174.49 (CO**C**H_{3b}), 173.5 (CO), 173.4 (CO), 171.8 (CO $\times$ 2), 61.2 (Ser-($C3_a$)), 61.1 (Ser-($C3_b$)), 55.7 (Ser-($C2_a$)), 55.6 (Ser-($C2_b$)), 54.97 (Cys-($C2_a$)), 54.95 (Cys-($C2_b$)), 25.3 (Cys-($C3_a$)), 25.2 (Cys-($C3_b$)), 21.8 (CO**C**H_{3a}), 21.7 (CO**C**H_{3b}). HRMS-ESI $[M+H]^+$ calculated for formula $C_8H_{15}N_2O_5S^+$, 251.0702; found 251.0705. IR (cm^{-1}): 3314, 2946, 1737, 1666, 1633, 1538.

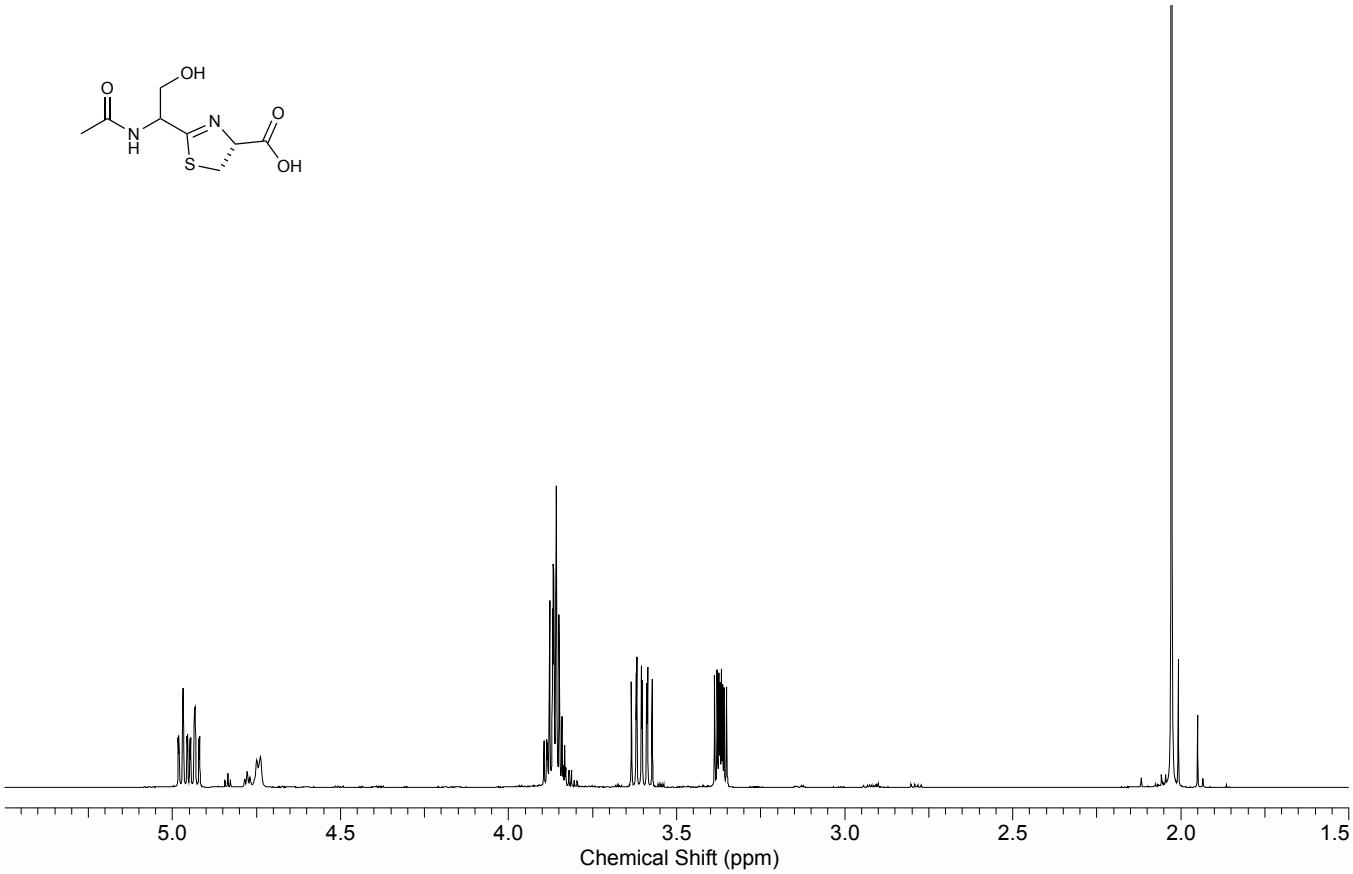


Fig. S77. ^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d, 1.50–5.50 ppm) spectrum of **Ac-Ser-thiazoline**.

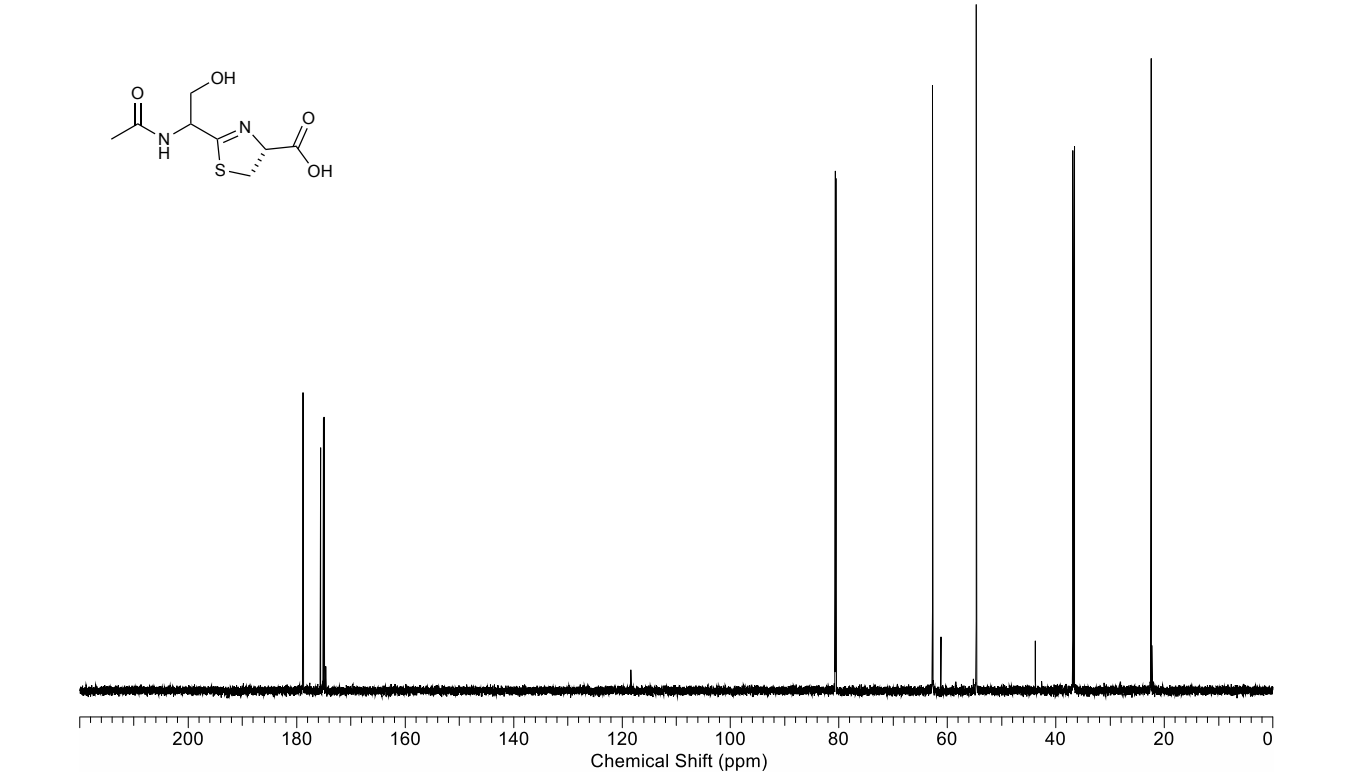


Fig. S78. ^{13}C NMR (176 MHz, H_2O/D_2O 9:1, 0–220 ppm) spectrum of **Ac-Ser-thiazoline**.

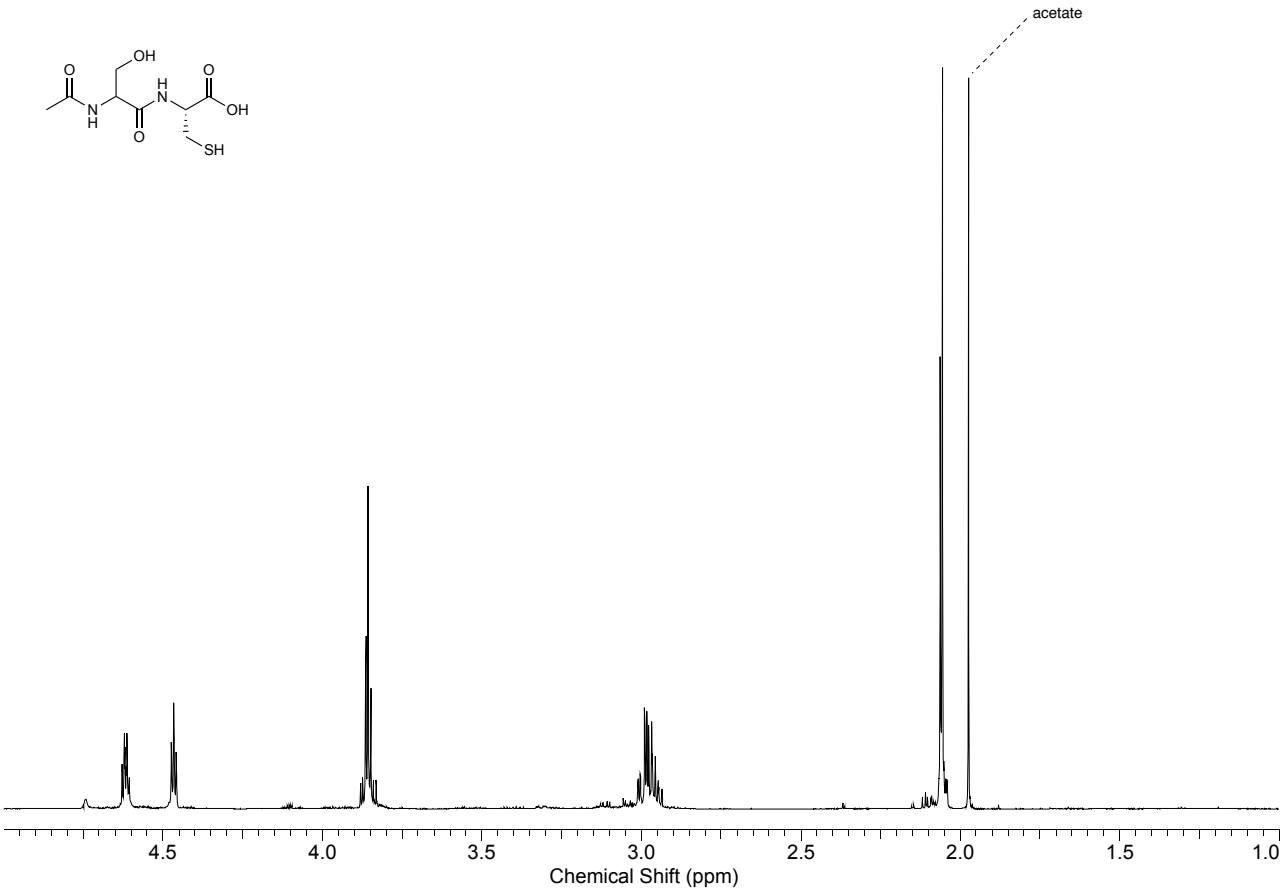


Fig. S79. ^{1}H NMR (700 MHz, D_2O, noesygppr1d, 1.0–5.**5** ppm) spectrum of **Ac-Ser-Cys-OH**.

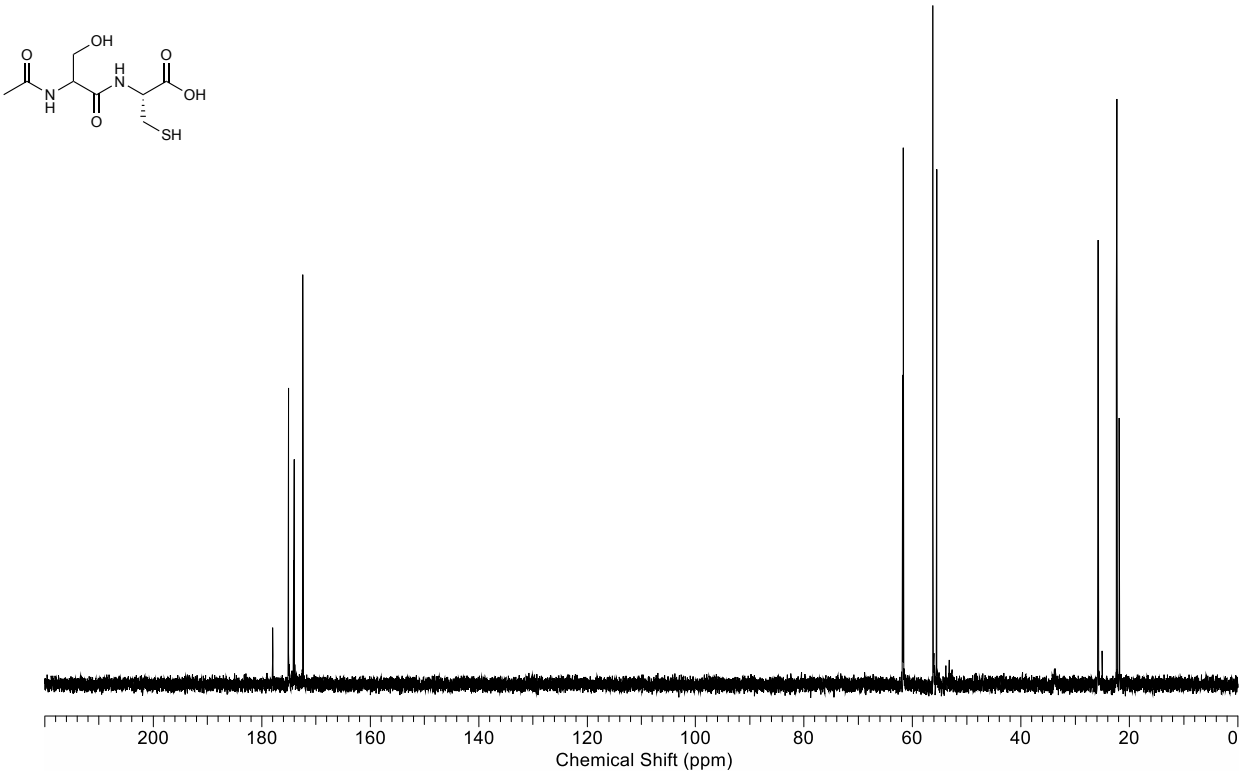
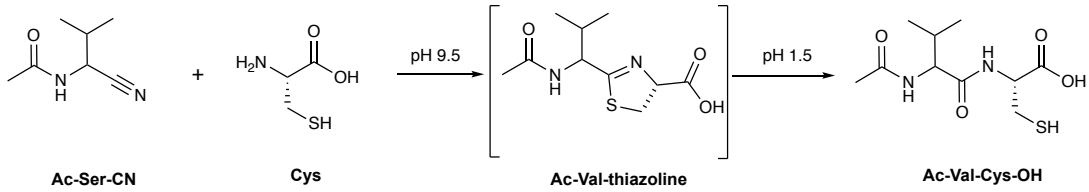


Fig. S80. ^{13}C NMR (176 MHz, D_2O, 0–200 ppm) spectrum of **Ac-Ser-Cys-OH**.

N-Acetyl-DL-valinyl-L-cysteine



A solution of *N*-acetyl-DL-valine nitrile (**Ac-Val-CN**; 302 mg, 2.15 mmol) and L-cysteine (**Cys**; 521 mg, 4.31 mmol) in H_2O/D_2O (9:1;10 mL) was adjusted to pH 9.5 and stirred at room temperature for 8 h. The formation of **Ac-Val-thiazoline** was observed. The solution was decreased to pH 1.5 and then stirred at room temperature for 12 h. The reaction mixture was concentrated *in vacuo* and the residue was purified by flash column chromatography (SiO_2; eluting with isopropanol/water 8:2) to yield 411 mg (1.57 mmol, 72%) as a diastereomeric mixture of **Ac-Val-Cys-OH** as a colourless solid.

*Data for **Ac-Val-thiazoline***

^{1}H NMR (700 MHz; D_2O, 2 diastereoisomers (50:50; a:b)): δ 4.68 - 4.61 (m, 2H, (C4)–H_a; (C4)–H_b), 4.22 (d, *J* = 6.7 Hz, 1H, Val-(C2)–H_a), 4.15 (d, *J* = 7.4 Hz, 1H, Val-(C2)–H_b), 3.31 (t, *J* = 10.7 Hz, 1H, (C5)–H_a), 3.26 (t, *J* = 10.4 Hz, 1H, (C5)–H_b), 3.12 - 3.04 (m, 2H, (C5)–H_a′; (C5)–H_b′), 1.85 - 1.76 (m, 2H, Val-(C3)–H_a; Val-(C3)–H_b), 1.73 (s, 3H, $COCH_{3a}$), 1.72 (s, 3H, $COCH_{3b}$), 0.65 - 0.58 (m, 12H, Val-(C4)–$H_{3a;}$ Val-(C4)–H_{3b}). ^{13}C NMR (176 MHz, D_2O, 2 diastereoisomers (50:50; a:b)): δ 178.8 (CO_2H_a), 178.5 (CO_2H_b), 177.1 ($C2_a$), 176.8 ($C2_b$), 175.0 (2 × $COCH_3$), 80.6 ($C4_a$), 80.3 ($C4_b$), 58.6 (Val-($C2_a$)), 58.6 (Val-($C2_b$)), 36.6 ($C5_a$), 36.3 ($C5_b$), 31.8 (Val-($C3_a$)), 31.7 (Val-($C3_b$)), 22.4 (2 × CO**C**H_3), 19.3 (Val-($C4_a$)), 19.3 (Val-($C4_b$)), 17.9 (Val-($C4_a$′)), 17.7 (Val-($C4_b$′)).

*Data for **Ac-Val-Cys-OH***

^{1}H NMR (700 MHz; D_2O, 2 diastereoisomers (44:55; a:b)): δ 4.52 (dd, *J* = 6.7, 4.7 Hz, 1H, Cys-(C2)–H_a), 4.50 (dd, *J* = 6.8, 4.7 Hz, 1H, Cys-(C2)–H_b), 4.09 (d, *J* = 6.9 Hz, 1H, Val-(C2)–H_a), 4.06 (d, *J* = 7.3 Hz, 1H, Val-(C2)–H_b), 2.94–2.87 (m, 4H, Cys–(C3)–H_2 × 2), 2.06–2.01 (m, 2H, Val-(C3)–H × 2), 1.99 (s, 3H, $COCH_{3a}$), 1.98 (s, 3H, $COCH_{3b}$), 0.90–0.89 (m, 12H, Val-(C4)–H_{3a}; Val-(C4)–H_{3b}). ^{13}C NMR (176 MHz, D_2O, 2 diastereoisomers (44:55; a:b)) (partial assignment): δ 174.42 (**C**OCH_{3a}), 174.38 (**C**OCH_{3b}), 173.8 (CO), 173.7 (CO), 173.68 (CO), 173.66 (CO), 59.70 (Val-($C2_a$)), 59.66 (Val-($C2_b$)), 55.14 (Cys-($C2_a$)), 55.10 (Cys-($C2_b$)), 30.1 (Val-($C3_a$)), 29.9 (Val-($C3_b$)), 25.23 (Cys-($C3_a$)), 25.21 (Cys-($C3_a$)), 21.63 (CO**C**H_{3a}), 21.56 (CO**C**H_{3b}), 18.4 (Val-($C4_a$)), 18.3 (Val-($C4_b$)), 17.6 (Val-($C4'_a$)), 17.4 (Val-($C4'_b$)). HRMS-ESI $[M+H]^+$ calculated for formula $C_{10}H_{19}N_2O_4S^+$, 263.1064; found 263.1066. IR (cm^{-1}): 3320, 3120, 3032, 2962, 1735, 1644, 1577, 1537.

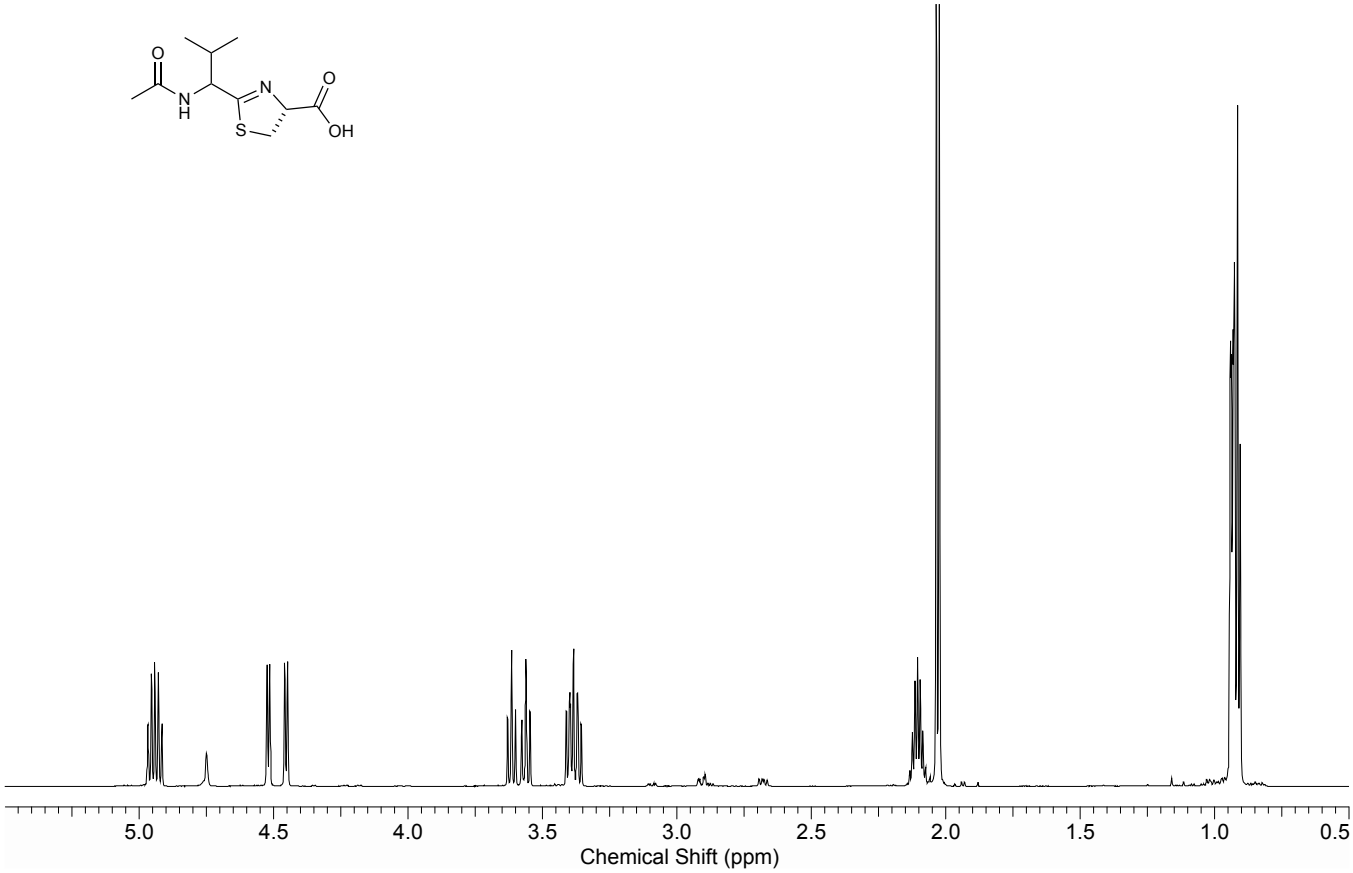


Fig. S81. ^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d, 0.50–5.50 ppm) spectrum of **Ac-Val-thiazoline**.

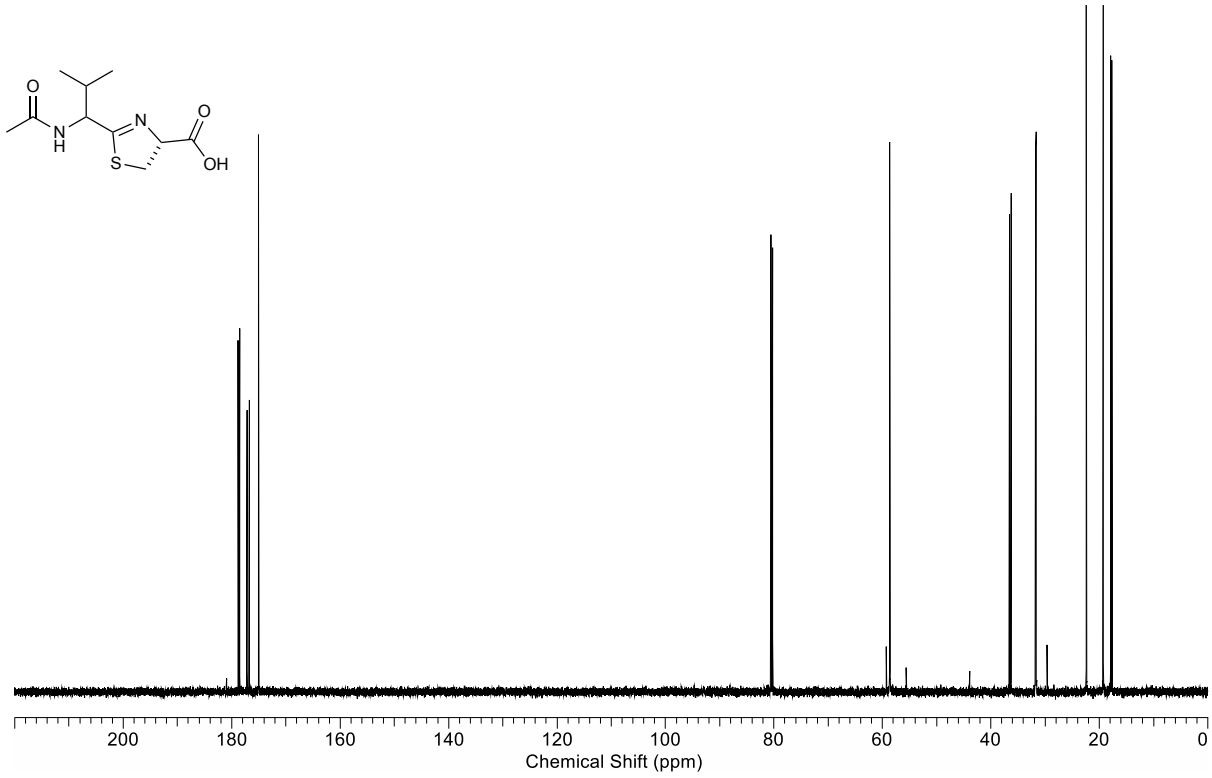


Fig. S82. ^{13}C NMR (176 MHz, H_2O/D_2O 9:1, 0–220 ppm) spectrum of **Ac-Val-thiazoline**.

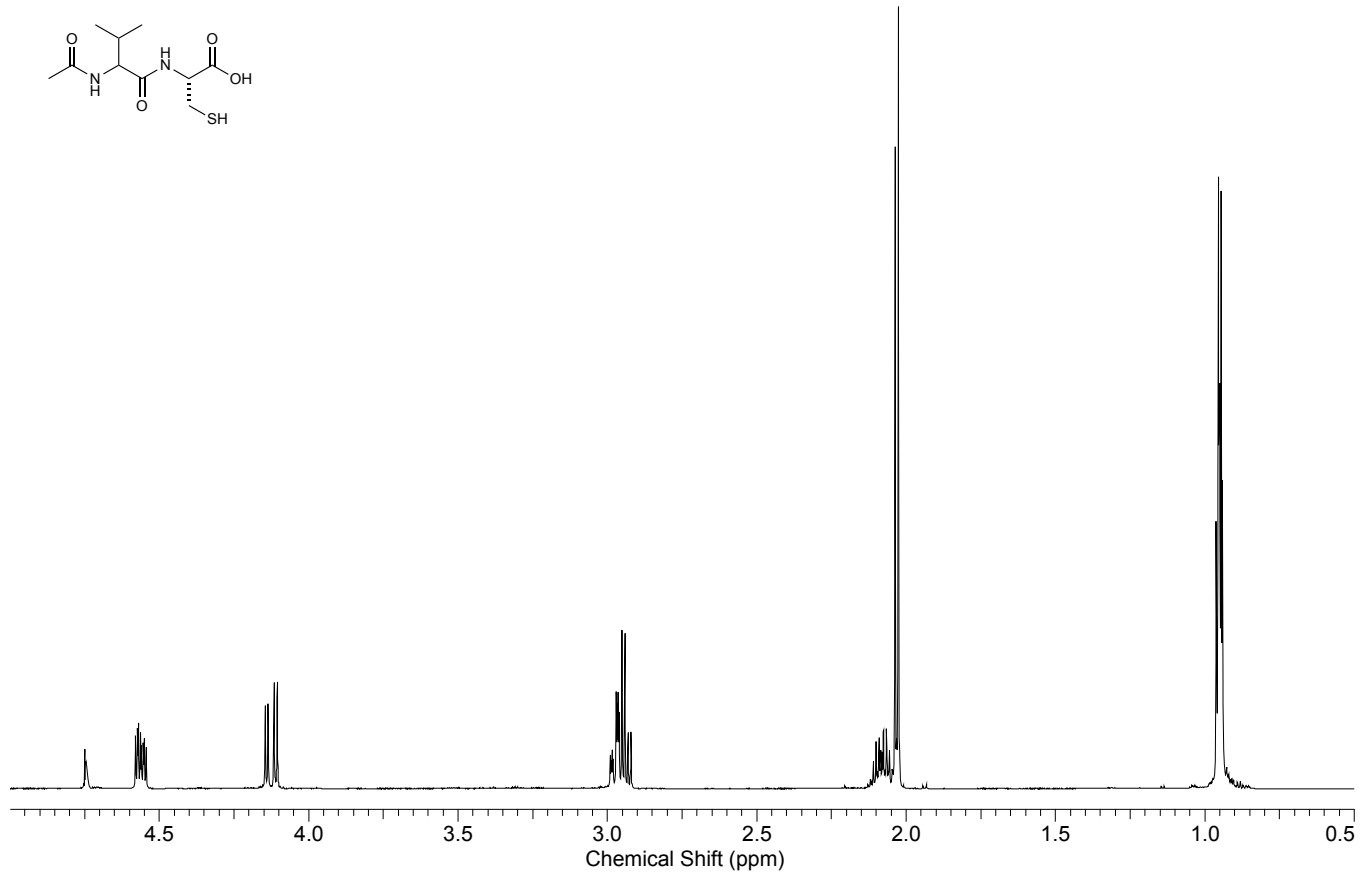


Fig. S83. ^{1}H NMR (700 MHz, D_2O, noesygppr1d, 0.5–5.0 ppm) spectrum of **Ac-Val-Cys-OH**.

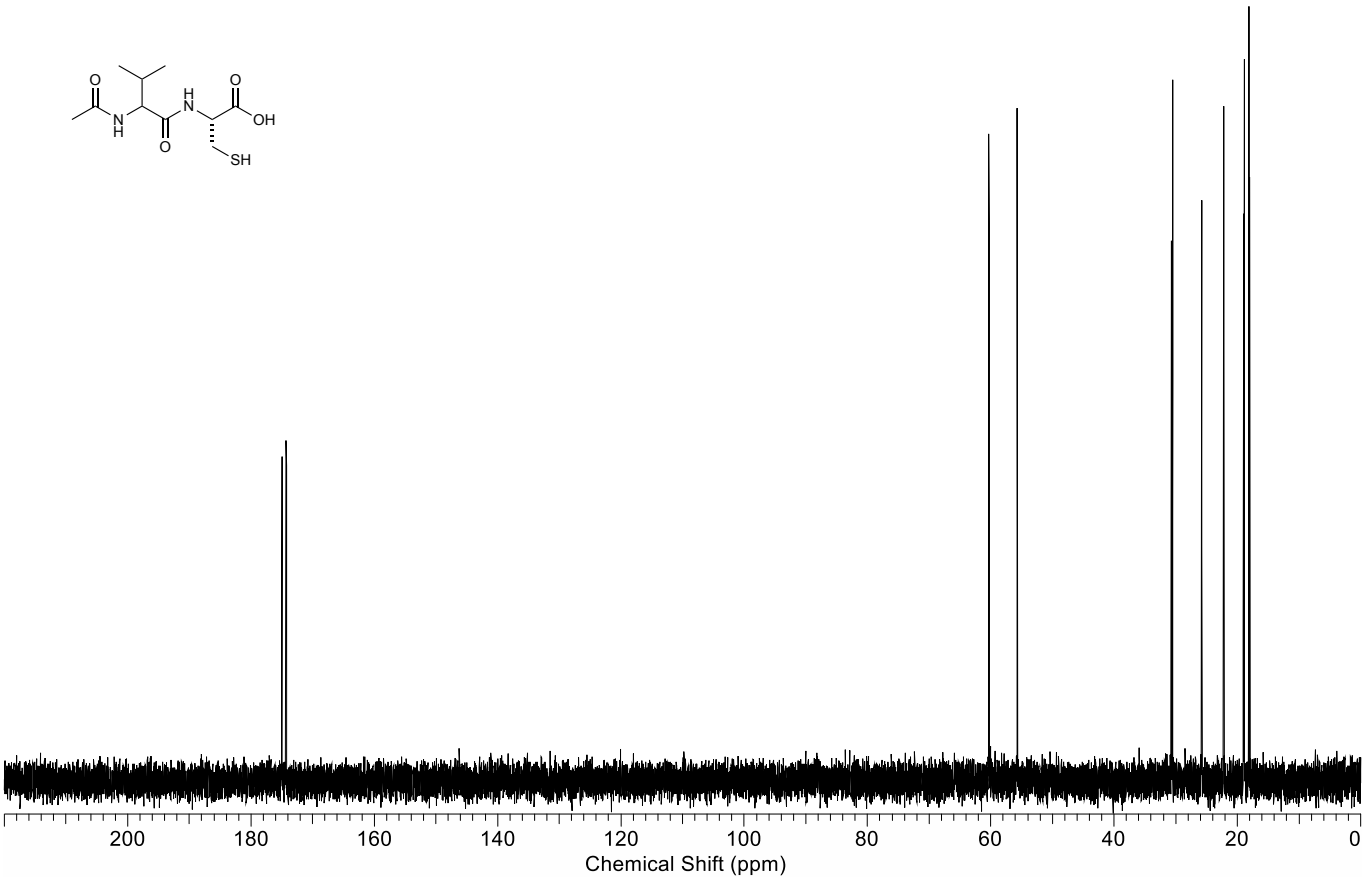
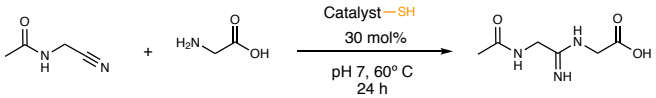


Fig. S84. ^{13}C NMR (176 MHz, D_2O, 0–200 ppm) spectrum of **Ac-Val-Cys-OH**.

Catalytic prebiotic peptide and amidine syntheses from α-amidonitriles

Catalyst screening for the coupling of N-acetylglycine nitrile with glycine



N-Acetylglycine nitrile **Ac-Gly-CN** (200 mM) (*5*), glycine **Gly** (200 mM), and thiol catalyst (30 mol%) were adjusted to pH 7 with 1 – 4 M HCl/NaOH and the solution was incubated at 60 °C for 24 h. The reaction was then analysed and quantified by NMR spectroscopy (^{1}H, ^{13}C, ^{1}H–^{13}C HMBC, ^{1}H–^{1}H COSY, and ^{1}H–^{13}C HSQC). Coupling yields for each thiol catalyst are given in Fig S85

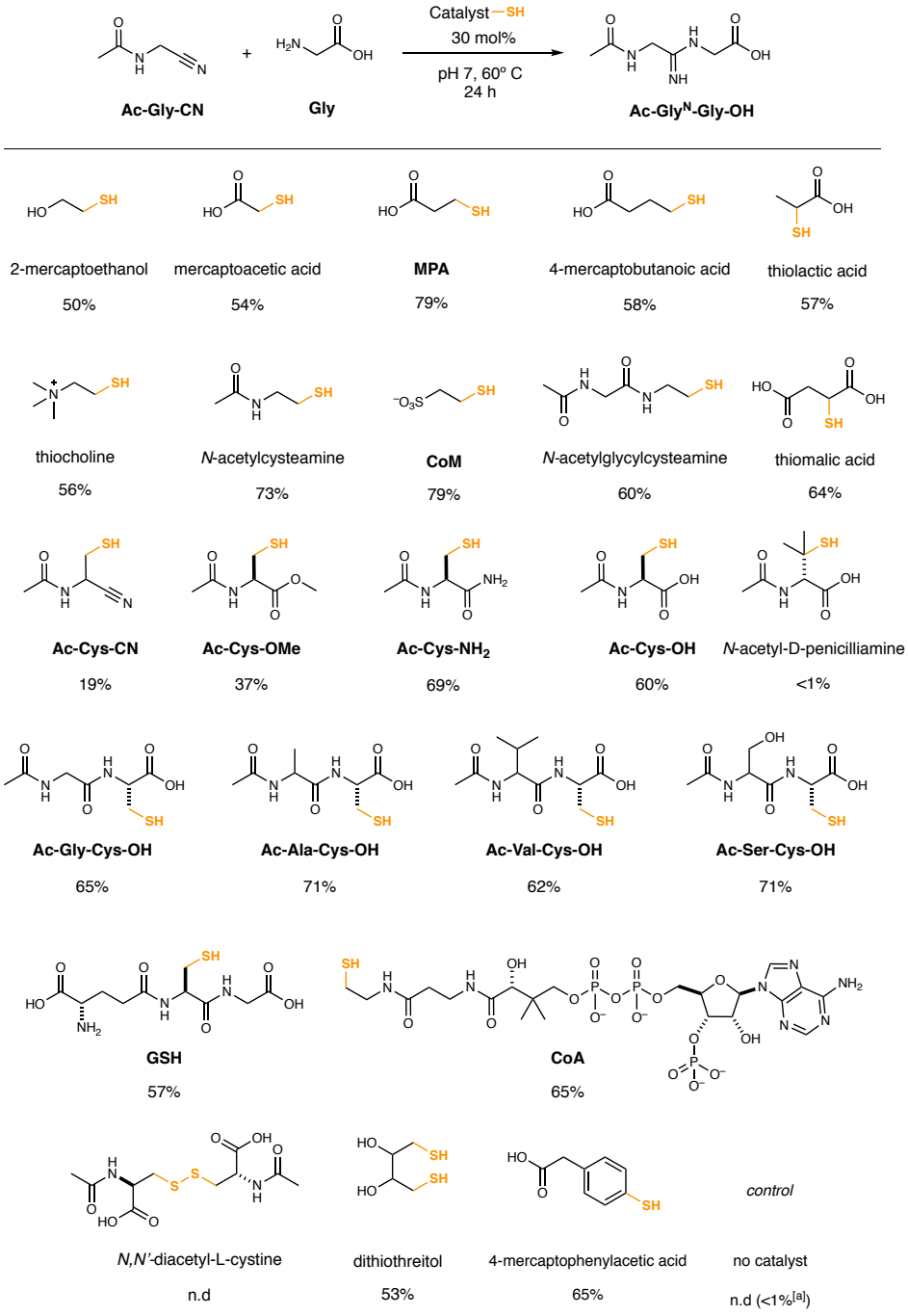
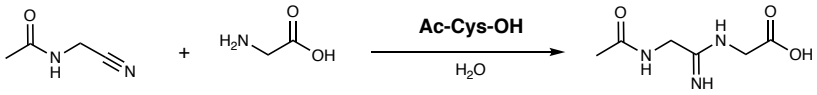


Fig S85. Yields for the formation of **Ac-Gly^{N}-Gly-OH** by thiol-catalyzed (Catalyst–SH; 30 mol%) coupling of **Ac-Gly-CN** (200 mM) and **Gly** (200 mM) after 24 h. n.d = not detected. [a]Coupling (<1%) only observed after 7 d. **MPA** = 3-mercaptopropionic acid; **CoA** = co-enzyme A; **GSH** = glutathione; **CoM** = co-enzyme M.

Optimisation of the coupling of N-acetylglycine nitrile with glycine catalysed by N-acetyl-L-cysteine



Ac-Gly-CN **Gly** **Ac-Gly^{N}-Gly-OH**

N-Acetylglycine nitrile **Ac-Gly-CN** (10 – 200 mM), glycine **Gly** (1 equiv.), and *N*-acetyl-L-cysteine (0.1–2.0 equiv) were adjusted to the specified pH with 1 – 4 M HCl/NaOH and the solution was incubated at the specified temperature for 24 h. The reaction was then analysed and quantified by NMR spectroscopy (^{1}H, ^{13}C, ^{1}H–^{13}C HMBC, ^{1}H–^{1}H COSY, and ^{1}H–^{13}C HSQC). Incubation of **Ac-Gly-CN** (200 mM) and glycine **Gly** (200 mM) with no catalyst at pH 7 and 60 °C yielded no observable **Ac-Gly^{N}-Gly-OH** after 1 d and <1% after 7 d (Fig. S86).

Ac-Gly-CN (mM)	Gly (mM)	Ac-Cys-OH (mol%)	Temp (ºC)	pH	Ac-Gly^{N}-Gly-OH (%)
200	200	0.3	rt	5.0	0
200	200	0.3	60	5.0	6
200	200	0.1	60	7.0	22
200	200	0.3	rt	7.0	28
200	200	0.3	60	7.0	60
200	200	0.3	rt	9.0	68[a]
200	200	0.3	40	9.0	70[b]
200	200	2.0	60	7.0	>95
200	200	-	60	7.0	n.d
100	100	0.3	60	7.0	62[c]
50	50	0.3	60	7.0	27
25	25	0.3	60	7.0	12
10	10	0.3	60	7.0	4

Table S5. Yields for *N*-acetyl-L-cysteine (**Ac-Cys-OH**) catalysed formation of of **Ac-Gly^{N}-Gly-OH** after the reaction of **Ac-Gly-CN** with **Gly** (1.0 eq.) at various pH, catalyst loadings, temperatures, after 24 h. n.d = not detectable after 24 h. [a] Combined yield of peptidyl amidine **Ac-Gly^{N}-Gly-OH** (63%) and peptide **Ac-Gly-Gly-OH** (5%). [b] Combined yield of peptidyl amidine **Ac-Gly^{N}-Gly-OH** (19%) and peptide **Ac-Gly-Gly-OH** (51%). [c] Combined yield of peptidyl amidine **Ac-Gly^{N}-Gly-OH** (56%) and peptide **Ac-Gly-Gly-OH** (6%).

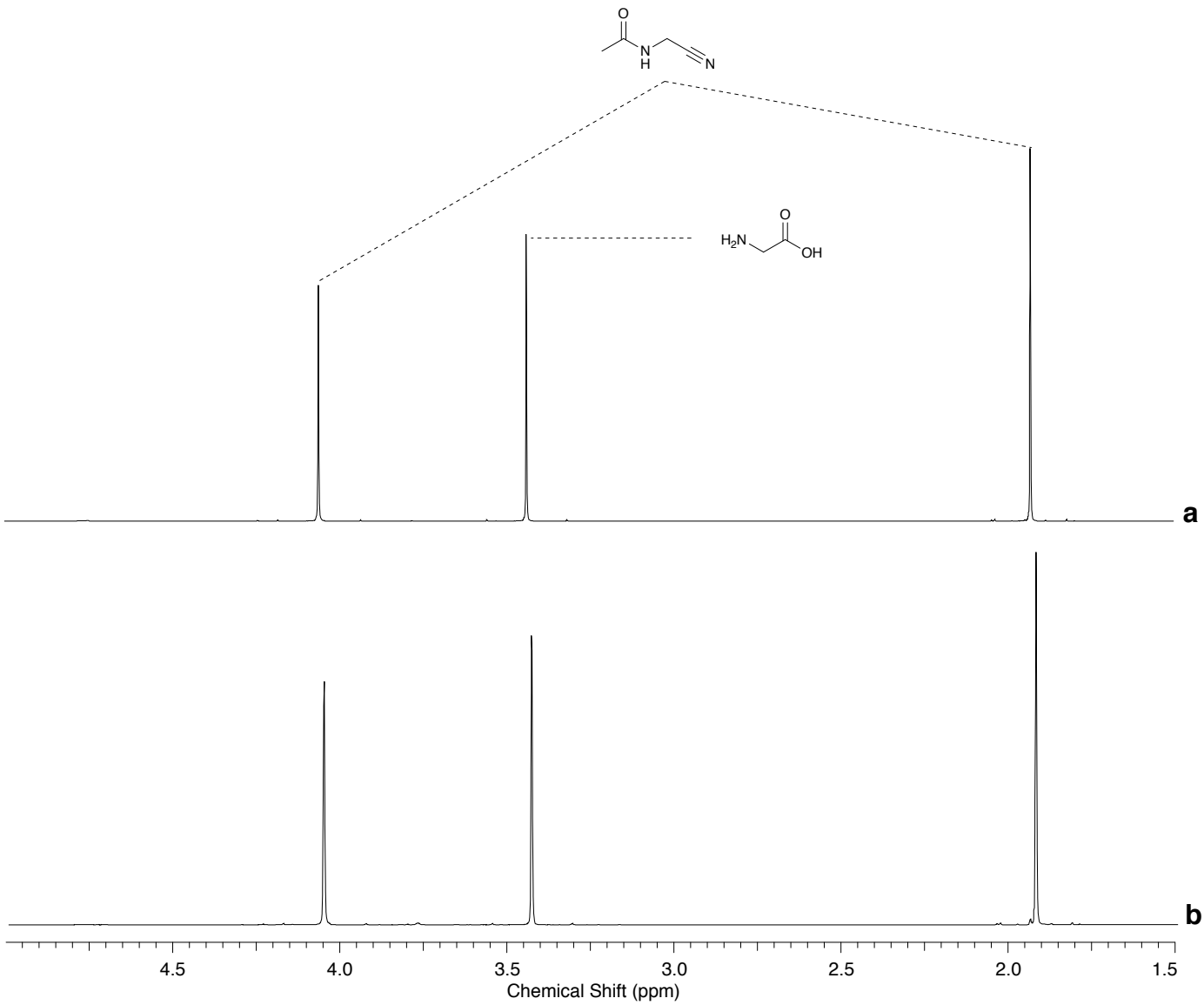
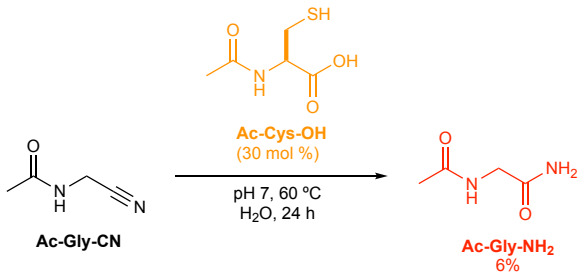


Fig. S86. ^{1}H NMR spectra (600 MHz, H_2O, noesygppr1d, 1.50–5.00 ppm) of **Ac-Gly-CN** (200 mM) and **Gly** (200 mM) incubated at pH 7 at 60 °C after: a) 24 h and b) 7 d. **Ac-Gly^{N}-Gly-OH** was not detectable after 24 h. **Ac-Gly^{N}-Gly-OH** (<1%) was observed only after 7 d.

Incubation of N-acetylglycine nitrile with N-acetyl-L-cysteine at pH 7 and 60 ℃



N-Acetylglycine nitrile **Ac-Gly-CN** (200 mM) and *N*-acetyl-L-cysteine **Ac-Cys-OH** (30 mol %) was dissolved in H_2O (1 mL) and the solution was adjusted to pH 7.0 with 4 M NaOH. The reaction was incubated at 60 °C for 24 h. The reaction was analysed by NMR spectroscopy. *N*-acetylglycinamide **Ac-Gly-NH₂** (6%) and partial aerial oxidation of **Ac-Cys-OH** to *N*,*N*′-diacetyl-L-cystine (10%) was observed.

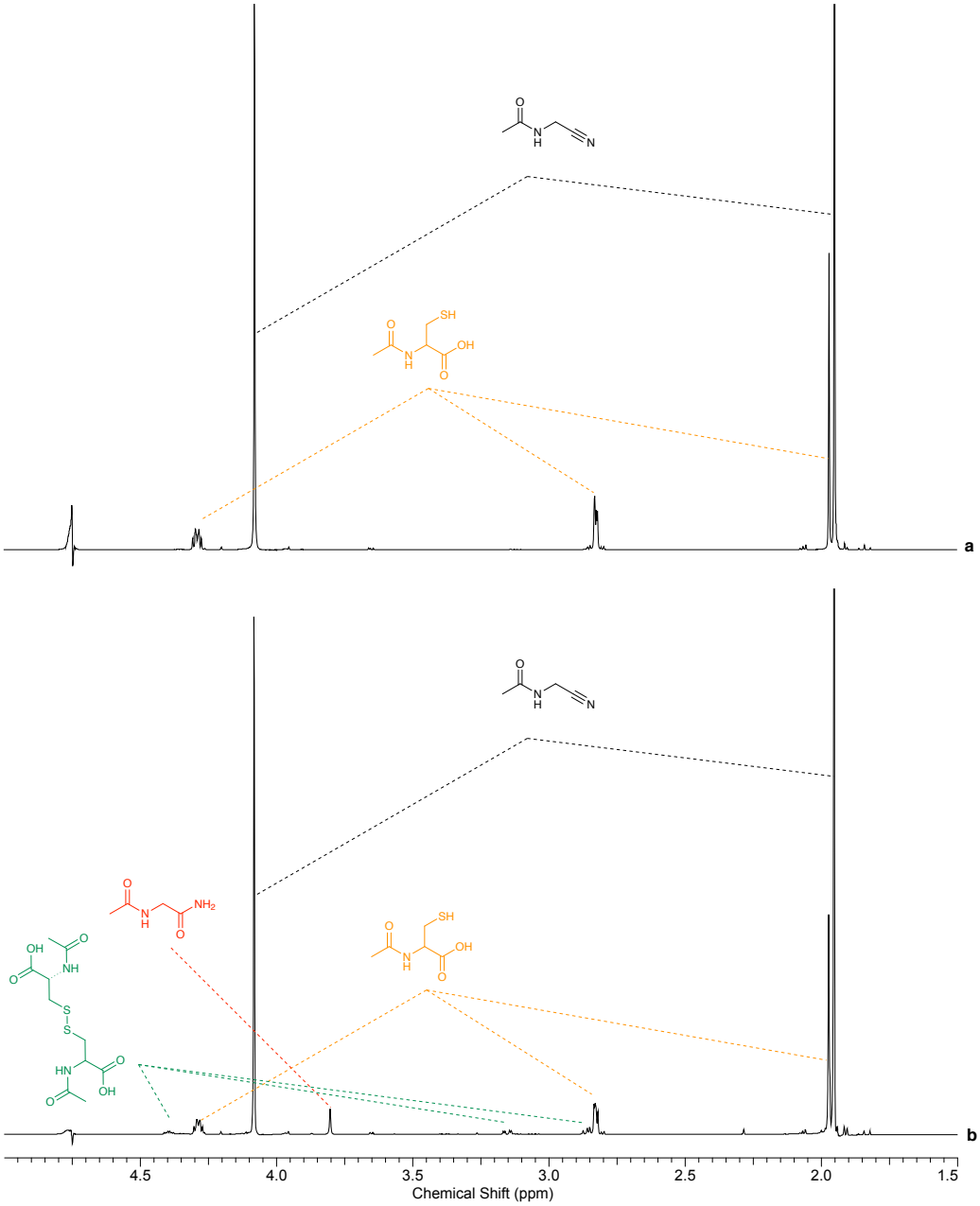
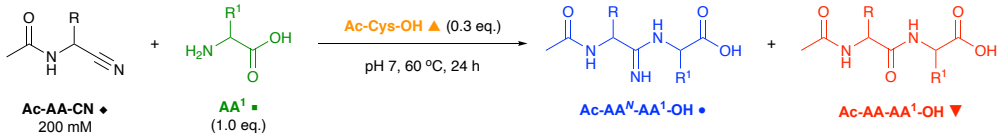


Fig. S87. ^{1}H NMR (600 MHz, H_2O, noesygppr1d, 1.50 – 5.00 ppm) spectrum to show *N*-acetylglycine nitrile **Ac-Gly-CN** (200 mM) and *N*-acetyl-L-cysteine **Ac-Cys-OH** (60 mM) at pH 7 **a.** before heating at 60 °C and **b**. after heating at 60 °C for 24 h.

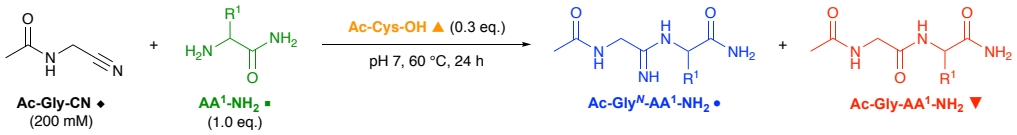
General prebiotic coupling procedures

N-Acetyl-L-cysteine catalysed coupling of N-acetylaminonitrile with α-amino acids



All α-amino acid **AA¹** couplings were carried out and analysed according to the following procedure unless stated otherwise. A solution of *N*-acetylaminonitrile (**Ac-AA-CN**, 200 mM), **AA¹** (1 – 2 equiv.), *N*-acetyl-L-cysteine (**Ac-Cys-OH**, 0.3 – 2.0 equiv.), and (methylsulfonyl)methane (MSM, 5 or 50 mM) in H_2O/D_2O (9:1) was adjusted to pH 7 with 0.1 – 4M HCl/NaOH. The solution was then incubated at room temperature or 60 °C for 24 h and analysed by high-resolution mass spectrometry and NMR spectroscopy (^1H, ^{13}C, ^1H–^1H COSY, ^1H–^{13}C HMBC, and ^1H–^{13}C HSQC). The products were quantified by ^1H NMR spectroscopy against the internal MSM standard. Yields of coupling are given in Table S6. α-Amino acids **AA¹** were all of L-configuration, unless stated otherwise with a modified stereochemical prefix (e.g DL-**AA¹** or D-**AA¹**).

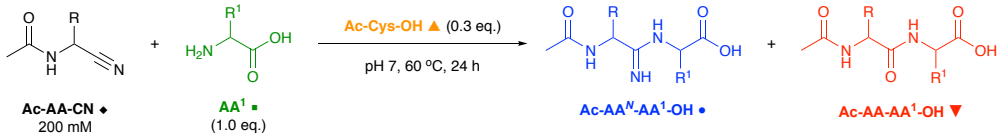
N-Acetyl-L-cysteine catalysed coupling of N-acetylglycine nitrile with α-amino amides



All α-amino amide **AA¹-NH₂** couplings were carried out and analysed according to the following procedure unless stated otherwise. A solution of *N*-acetylglycine nitrile (**Ac-Gly-CN**, 200 mM), **AA¹-NH₂** (200 mM), *N*-acetyl-L-cysteine (**Ac-Cys-OH**, 60 mM), and MSM (5 or 50 mM) in H_2O/D_2O (9:1) was adjusted to pH 7 with 0.1 – 4M HCl/NaOH. The solution was incubated at 60 °C for 24 h and analysed by high-resolution mass spectrometry and NMR spectroscopy (^1H, ^{13}C, ^1H–^1H COSY, ^1H–^{13}C HMBC, and ^1H–^{13}C HSQC). The products were quantified by ^1H NMR spectroscopy against the internal MSM standard. Yields are given in Table S7. α-Amino amides **AA¹-NH₂** were all of L- configuration, except for D-valinamide (D-**Val-NH₂**), D-leucinamide (D-**Leu-NH₂**), and D-alaninamide (D-**Ala-NH₂**).

Supplementary table of coupling yields and high-resolution mass spectrometry data

*Coupling of N-acetylaminonitrile **Ac-AA-CN** with α-amino acids **AA¹** at pH 7 and 60 °C*



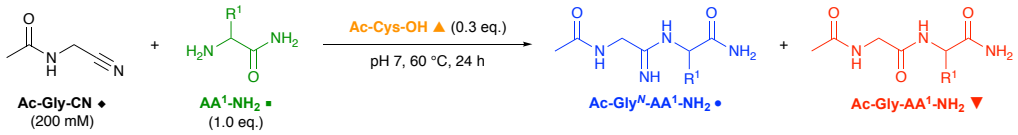
Ac-AA-CN ♦ 200 mM + **AA¹** ■ (1.0 eq.) → **Ac-AA^N-AA¹-OH** ● + **Ac-AA-AA¹-OH** ▼

Ac-AA-CN ♦	AA¹ ■	Yield (%)		HRMS-ESI for Ac-AA^N-AA¹-OH		
		Ac-AA^N-AA¹-OH ●	Ac-AA-AA¹-OH▼	Formula	Theoretical	Found
Ala	Gly	60 (>95[a])	2	$C_7H_{14}N_3O_3$ [M+H]$^+$	188.1029	188.1033
Ala	Ala	25 (83[b])	-	$C_8H_{16}N_3O_3$ [M+H]$^+$	202.1186	202.1182
Gly	Gly	60 (>95[c])	-	$C_6H_{12}N_3O_3$ [M+H]$^+$	174.0873	174.0873
Gly	DL-Ala	43 (79[d])	-	$C_7H_{14}N_3O_3$ [M+H]$^+$	188.1030	188.1028
Gly	Arg	37 (78[e])	-	$C_{10}H_{21}N_6O_3$ [M+H]$^+$	273.1670	273.1667
Gly	Asn	9	45[f]	$C_8H_{15}N_4O_4$ [M+H]$^+$	231.1088	231.1086
Gly	Asp	58	-	$C_8H_{14}N_3O_5$ [M+H]$^+$	232.0928	232.0925
Gly	Gln	56	-	$C_9H_{17}N_4O_4$ [M+H]$^+$	245.1244	245.1243
Gly	Glu	58	-	$C_9H_{16}N_3O_5$ [M+H]$^+$	246.1084	246.1087
Gly	His	73	-	$C_{10}H_{16}N_5O_3$ [M+H]$^+$	254.1248	254.1257
Gly	Ile	55	-	$C_{10}H_{20}N_3O_3$ [M+H]$^+$	230.1499	230.1498
Gly	Leu	53	-	$C_{10}H_{20}N_3O_3$ [M+H]$^+$	230.1499	230.1498
Gly	Lys	70[g]	-	$C_{10}H_{21}N_4O_3$ [M+H]$^+$	245.1608	245.1609
Gly	DL-Met	72	-	$C_9H_{18}N_3O_3S$ [M+H]$^+$	248.1063	248.1064
Gly	Phe	21 (52[h])	-	$C_{13}H_{18}N_3O_3$ [M+H]$^+$	264.1343	264.1338
Gly	Pro	58	-	$C_9H_{16}N_3O_3$ [M+H]$^+$	214.1186	214.1188
Gly	Ser	-	61 (74[i])	$C_7H_{12}N_2O_5Na$ [M+Na]$^+$	226.0638[j]	226.0637
Gly	Thr	-	51 (80[k])	$C_8H_{14}N_2O_5Na$ [M+Na]$^+$	241.0795[l]	241.0792
Gly	Trp	32	5	$C_{15}H_{19}N_4O_3$ [M+H]$^+$	303.1452	303.1447
Gly	Tyr[m]	20	-	$C_{13}H_{18}N_3O_4$ [M+H]$^+$	280.1292	280.1294
Gly	Val	42 (79[n])	6	$C_9H_{18}N_3O_3$ [M+H]$^+$	216.1343	216.1345
Glx	Gly	33 (56[o])	-	$C_9H_{15}N_4O_3$ [M+H]$^+$	227.1141	227.1144
Ser	Gly	61 (90[p])	-	$C_7H_{13}N_3O_4Na$ [M+Na]$^+$	226.0798	226.0801
Ser	Ala	25 (71[q])	-	$C_8H_{16}N_3O_4$ [M+H]$^+$	218.1135	218.1136
Val	Gly	3 [(27[r]), (44[s]), (79[t])]	-	$C_9H_{18}N_3O_3$ [M+H]$^+$	216.1343	216.1342

Table S6. Yields and ESI-HRMS data for *N*-acetyl-L-cysteine (**Ac-Cys-OH** ▲ (0.3 eq.)) catalysed formation of **Ac-AA^N-AA¹-OH** ● and **Ac-AA-AA¹-OH** ▼ from the reaction of **Ac-AA-CN** (200 mM) with **AA¹** ■ (1.0 eq.) at pH 7, 60 °C after 24 h, unless stated otherwise. - = not observed.

[a] Reaction of **Ac-Ala-CN** (200 mM), **Gly** (400 mM), **Ac-Cys-OH** (400 mM) at pH 7 and 60 °C for 24 h.
[b] Reaction of **Ac-Ala-CN** (200 mM), **Ala** (400 mM), **Ac-Cys-OH** (400 mM) at pH 7 and 60 °C for 24 h.
[c] Reaction of **Ac-Gly-CN** (200 mM), **Gly** (400 mM), **Ac-Cys-OH** (400 mM) at pH 7 and 60 °C for 24 h.
[d] Reaction of **Ac-Gly-CN** (200 mM), DL-**Ala** (600 mM), **Ac-Cys-OH** (60 mM) at pH 7 and 60 °C for 24 h.
[e] Reaction of **Ac-Gly-CN** (200 mM), **Arg** (400 mM), **Ac-Cys-OH** (60 mM) at pH 7 and 60 °C for 24 h.
[f] *C*-terminal succinimide formation (<5%) was observed as an additional product. Succinimide formation of asparaginyl peptides is well documented (*49*).
[g] Combined yield for the N^2, N^6, and N^2,N^6-bis-amidines coupling products (***N*²-(Ac-Gly^N)-Lys-OH** (43%), ***N*⁶-(Ac-Gly^N)-Lys-OH** (24%) and ***N*²,*N*⁶-bis(Ac-Gly^N)-Lys-OH** (3%)). See Fig. S108.
[h] Reaction of **Ac-Gly-CN** (200 mM), **Phe** (400 mM), **Ac-Cys-OH** (60 mM) at pH 7 and 60 °C for 24 h.
[i] Reaction of **Ac-Gly-CN** (200 mM), **Ser** (200 mM) and **MPA** (3-mercaptopropionic acid; 200 mM) as the catalyst instead of **Ac-Cys-OH** (60 mM) at pH 7 and 60 °C for 48 h.
[j] ESI-HRMS data given for **Ac-Gly-Ser-OH**.
[k] Reaction of **Ac-Gly-CN** (200 mM), **Thr** (200 mM) and **MPA** (3-mercaptopropionic acid; 200 mM) as the catalyst instead of **Ac-Cys-OH** at pH 7 and 60 °C for 48 h.
[l] ESI-HRMS data given for **Ac-Thr-Ser-OH**.
[m] L-Tyrosine **Tyr** exhibits extremely low solubility in water. See Reference 50 and 51 for a reported solubility of **Tyr** in water, No further attempts were made to optimise **Tyr** coupling.
[n] Reaction of **Ac-Gly-CN** (200 mM), **Val** (400 mM), **Ac-Cys-OH** (60 mM) at pH 7 and 60 °C for 24 h.
[o] Reaction of *N*-acetyl-2-aminoglutaronitrile **Ac-Glx-CN** (200 mM), **Gly** (200 mM), **Ac-Cys-OH** (60 mM) at pH 7 and 60 °C for 4 d.
[p] Reaction of **Ac-Ser-CN** (200 mM), **Gly** (400 mM), **Ac-Cys-OH** (400 mM) at pH 7 and 60 °C for 24 h.
[q] Reaction of **Ac-Ser-CN** (200 mM), **Ala** (400 mM), **Ac-Cys-OH** (400 mM) at pH 7 and 60 °C for 24 h.
[r] Reaction of **Ac-Val-CN** (200 mM), **Gly** (400 mM), and **MPA** (3-mercaptopropionic acid; 400 mM) as the catalyst instead of **Ac-Cys-OH**, at pH 7 and 60 °C for 24 h.
[s] Reaction of **Ac-Val-CN** (200 mM), **Gly** (400 mM), and **MPA** (3-mercaptopropionic acid; 400 mM) as the catalyst instead of **Ac-Cys-OH**, at pH 7 and 60 °C for 48 h.
[t] Reaction of **Ac-Val-CN** (200 mM), **Gly** (400 mM), and **MPA** (3-mercaptopropionic acid; 400 mM) as the catalyst instead of **Ac-Cys-OH**, at pH 7 and 60 °C for 96 h.

Coupling of N-acetylglycine nitrile ***Ac-Gly-CN*** *with α-amino amides* ***AA^1-NH$_2$*** *at pH 7 and 60 °C*



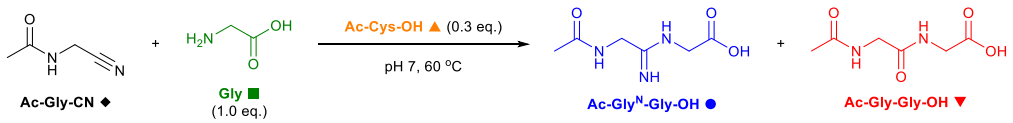
AA^1-NH$_2$ ■	**% Yield**		**HRMS-ESI for Ac-Gly-AA^1-NH$_2$**		
	Ac-Gly^N-AA^1-NH$_2$ ●	**Ac-Gly-AA^1-NH$_2$▼**	Formula	Theoretical	Found
Gly	21	52	$C_6H_{11}N_3O_3Na$ $[M+Na]^+$	196.0693	196.0689
D-Ala	3	63	$C_7H_{13}N_3O_3Na$ $[M+Na]^+$	210.0849	210.0851
Arg	14	56	$C_{10}H_{21}N_6O_3$ $[M+H]^+$	273.1670	273.1672
Asn[a]	-	72	$C_8H_{14}N_4O_4Na$ $[M+Na]^+$	253.0907	253.0910
Asp	6	58	$C_8H_{14}NO_5$ $[M+H]^+$	232.0928	232.0950
Gln	-	43	$C_9H_{16}N_4O_4Na$ $[M+Na]^+$	267.1064	267.1063
Glu	-	64	$C_9H_{15}N_3O_5Na$ $[M+Na]^+$	268.0904	268.0908
His	-	67	$C_{10}H_{15}N_5O_3$ $[M+H]^+$	254.1248	254.1241
Ile	12	47	$C_{10}H_{19}N_3O_3$ $[M+H]^+$	230.1499	230.1499
D-Leu	5	54	$C_{10}H_{20}N_3O_3$ $[M+H]^+$	230.1499	230.1508
Lys	25[b]	52[c]	$C_{10}H_{21}N_4O_3$ $[M+H]^+$	245.1608	245.1600
Met	5	62	$C_9H_{18}N_3O_3S$ $[M+H]^+$	248.1063	248.1066
Phe	8	52	$C_{13}H_{18}N_3O_3$ $[M+H]^+$	264.1343	264.1349
Pro	-	21 (67[d])	$C_9H_{15}N_3O_3Na$ $[M+Na]^+$	236.1006	236.1005
Ser	-	68[e] (75[f])	$C_7H_{14}N_3O_4$ $[M+H]^+$	204.0979	204.0978
Thr	-	69[g] (85[h])	$C_8H_{15}N_3O_4Na$ $[M+Na]^+$	240.0955	240.0960
Trp	4	45	$C_{15}H_{18}N_4O_3Na$ $[M+Na]^+$	325.1271	325.1272
Tyr	3	62	$C_{13}H_{17}N_3O_4Na$ $[M+Na]^+$	302.1111	302.1116
D-Val	7	50	$C_9H_{17}N_3O_3Na$ $[M+Na]^+$	238.1162	238.1158

Table S7. Yields and ESI-HRMS data for *N*-acetyl-L-cysteine (**Ac-Cys-OH** ▲ (0.3 eq.)) catalysed formation of **Ac-Gly^N-AA^1-OH** ● and **Ac-Gly-AA^1-OH** ▼ from the coupling of **Ac-Gly-CN** (200 mM) with **AA^1-NH$_2$** ■ (1.0 eq.) at pH 7, 60 °C, after 24 hours, unless stated otherwise. - = not observed.

[a] Reaction carried out with **Ac-Gly-CN** (200 mM), **Asn-NH$_2$** (200 mM) and **Ac-Cys-OH** (5 equiv.), pH 7 and 60 °C for 24 h.
[b] Reported amidine product is the N^6 coupling product only (***N*^{6}-(Ac-Gly^N)-Lys-NH$_2$**). Bisamidination was not detectable by ^{1}H NMR spectroscopy. See Fig. S158.
[c] Reported amide product is the N^2 coupling product (***N*^{2}-(Ac-Gly)-Lys-NH$_2$**). Bisacylation was not detectable by ^{1}H NMR spectroscopy. See Fig. S158.
[d] Reaction carried out with **Ac-Gly-CN** (200 mM), **Pro-NH$_2$** (400 mM) and **Ac-Cys-OH** (5 equiv.), pH 7 and 60 °C for 24 h.
[e] An intermediate oxazoline, (2-(acetamidomethyl)-4,5-dihydrooxazole-4-carboxamide (6%), was observed. See Fig. S166.
[f] Reaction of **Ac-Gly-CN** (200 mM), **Ser-NH$_2$** (200 mM) and **MPA** (3-mercaptopropionic acid; 200 mM) as the catalyst instead of **Ac-Cys-OH** at pH 7 and 60 °C for 48 h.
[g] An intermediate oxazoline (2-(acetamidomethyl)-5-methyl-4,5-dihydrooxazole-4-carboxamide (<5%) was observed. See Fig. S167.
[h] Reaction of **Ac-Gly-CN** (200 mM), **Thr-NH$_2$** (200 mM) and **MPA** (3-mercaptopropionic acid; 200 mM) as the catalyst instead of **Ac-Cys-OH** at pH 7 and 60 °C for 48 h.

Characterisation of coupling reactions of N-acetyl-L-cysteine-catalysed coupling of N-acetylglycine nitrile and α-amino acids

Coupling of N-acetylglycine nitrile ***Ac-Gly-CN*** *with glycine* ***Gly*** *at pH 7 and 60 °C*



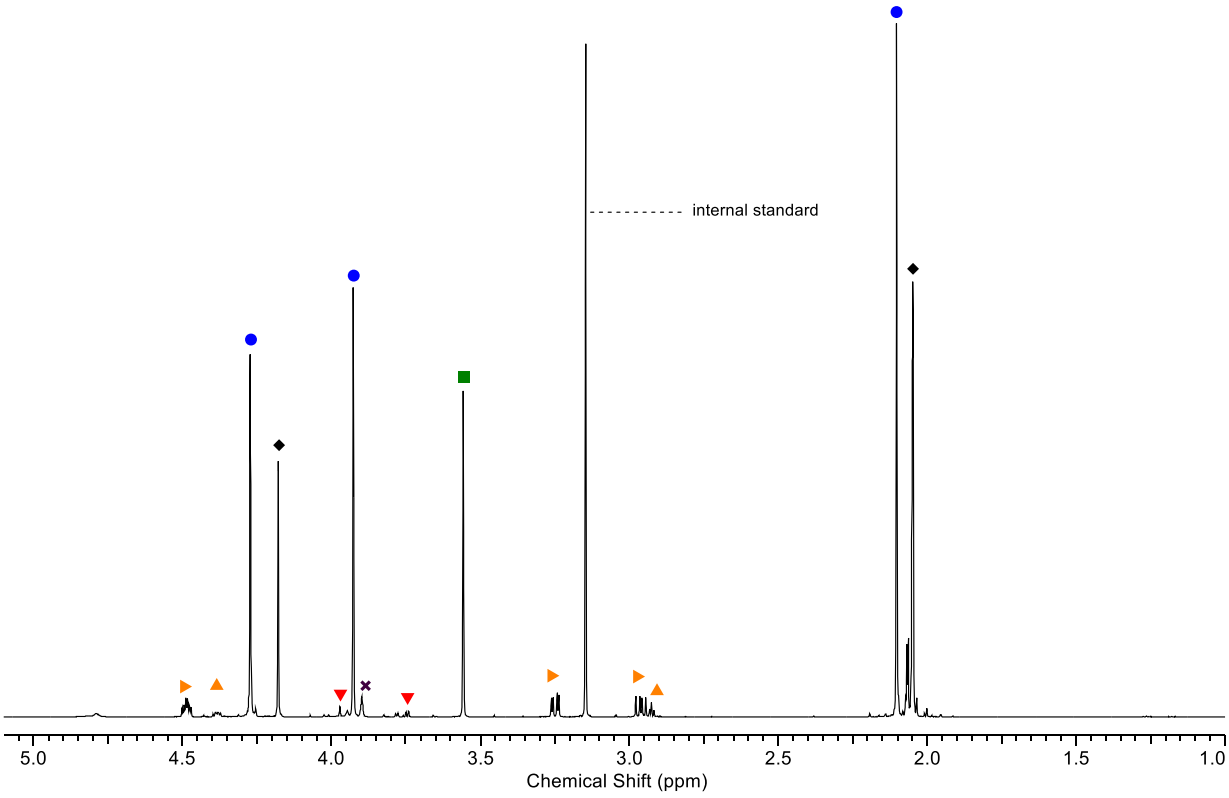


Fig. S88. ^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d, 1.0-5.0 ppm) spectrum to show the reaction of *N*-acetyl glycine nitrile (**Ac-Gly-CN**, 200 mM) with glycine (**Gly**, 200 mM) and (**Ac-Cys-OH**, 60 mM, 0.3 eq.), with MSM (50 mM) as the internal standard, after 24 h at pH 7 and 60 °C. ▶ = *N,N'*-diacetyl-L-cystine (formed by aerial oxidation of **Ac-Cys-OH**) and ✖= *N*-acetylglycinamide, **Ac-Gly-NH$_2$**

^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d) *(2-acetamido-1-iminoethyl)glycine*, **Ac-Gly^{N}-Gly-OH** (●): δ_H 4.27 (2H, s, AcNHC*H*$_2$), 3.93 (2H, s, C*H*$_2$COOH), 2.10 (3H, s, *H*$_3$C(CO)); *N-Acetylglycylglycine*, **Ac-Gly-Gly-OH** (▼) (partial assignment): δ_H 3.97 (2H, s, AcNHC*H*$_2$), 3.74 (2H, AB obs., C*H*$_2$COOH); *Glycine*, **Gly** (■): δ_H 3.56 (2H, s, C*H*$_2$); *N-Acetylglycinamide*, **Ac-Gly-NH$_2$** (✖) (partial assignment): δ_H 3.90 (2H, s, C*H*$_2$).

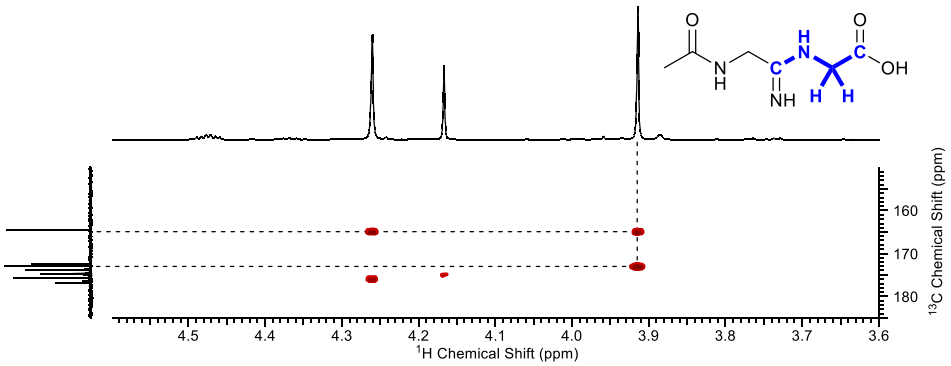
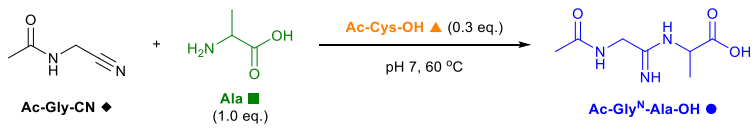


Fig. S89. ^{1}H–^{13}C HMBC (^{1}H: 700 MHz [3.50-4.50 ppm], ^{13}C: 176 MHz [150-185 ppm], H_2O/D_2O 9:1) spectrum showing the diagnostic $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling of **Gly**-αH-COOH in **Ac-Gly^{N}-Gly-OH** at 3.93 ppm with two resonances at 176 and 164 ppm, which is characteristic of amidine bond formation of **Gly**. See Fig. S88 for expanded and labelled ^{1}H NMR spectrum.

Coupling of N-acetylglycine nitrile ***Ac-Gly-CN*** *with DL-alanine DL-****Ala*** *at pH 7 and 60 ℃*



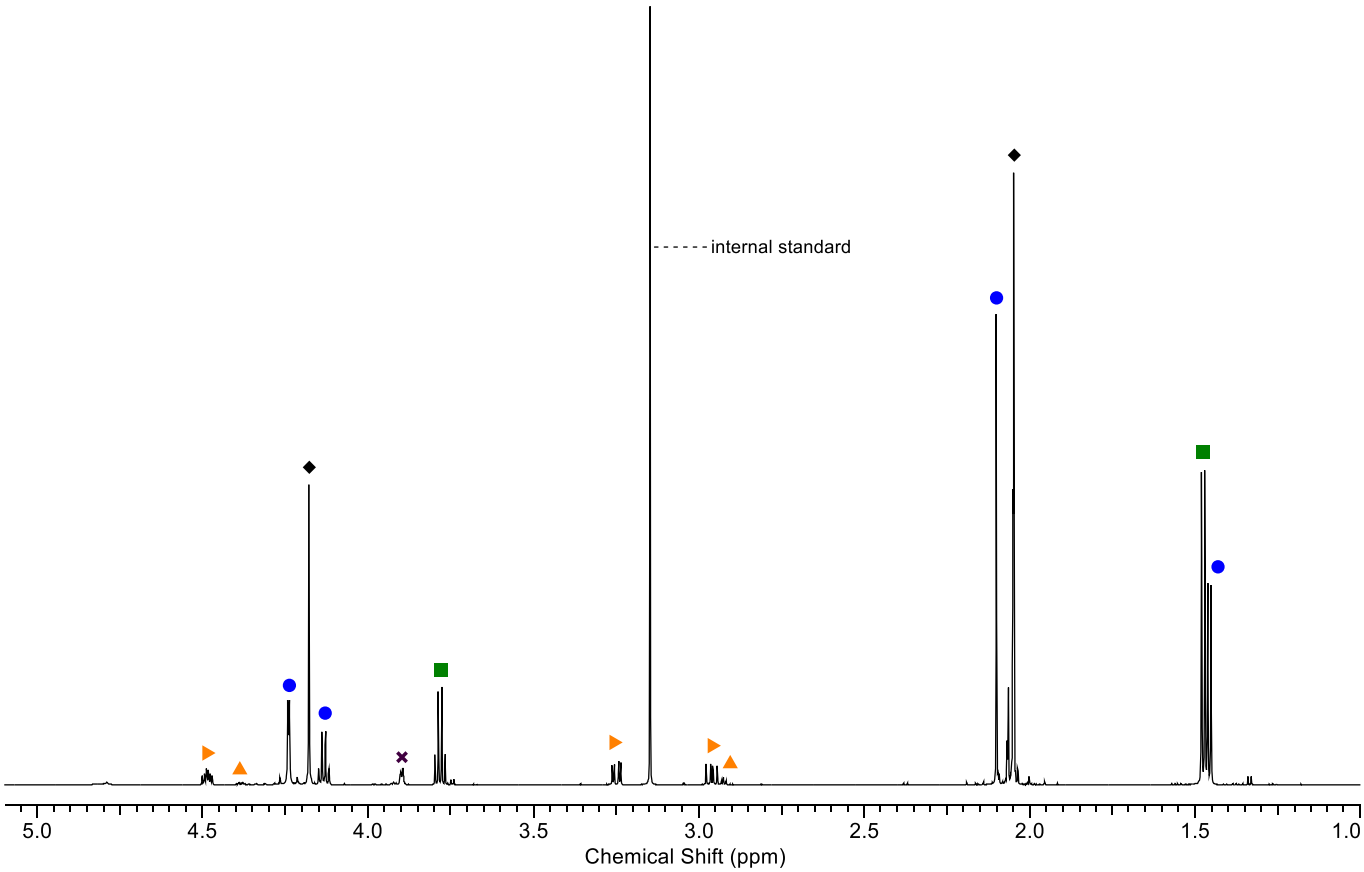


Fig. S90. ^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d, 1.0–5.0 ppm) spectrum to show the reaction of *N*-acetyl glycine nitrile (**Ac-Gly-CN**, 200 mM) with DL-alanine (DL-**Ala**, 200 mM) and *N*-acetyl-L-cysteine (**Ac-Cys-OH**, 60 mM, 0.3 eq.) with MSM (50 mM) as the internal standard after 24 h at pH 7 and 60 °C. ▶ = *N,N'*-diacetyl-L-cystine (formed by aerial oxidation of **Ac-Cys-OH**) and ✖= *N*-acetylglycinamide, **Ac-Gly-NH$_2$**

^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d) *(2-acetamido-1-iminoethyl)-D,L-alanine*, **Ac-Gly^{N}-Ala-OH** (●): δ_H 4.26 (1H, AB, *J* = 17.2 Hz, AcNHCH*H*), 4.22 (1H, AB, *J* = 17.2 Hz, AcNHCH*H*), 4.13 (1H, q, *J* = 7.2 Hz, C*H*(CH_3)), 2.10 (3H, s, *H*$_3$C(CO)), 1.46 (3H, d, *J* = 7.2 Hz, CH(C*H*$_3$)); *DL-Alanine*, *DL*-**Ala** (■): δ_H 3.78 (1H, q, *J* = 7.2 Hz, C*H*(CH_3)), 1.47 (3H, d, *J* = 7.2 Hz, CH(C*H*$_3$)); *N-Acetylglycinamide*, **Ac-Gly-NH$_2$** (✖) (partial assignment): δ_H 3.89 (2H, app. d., C*H*$_2$).

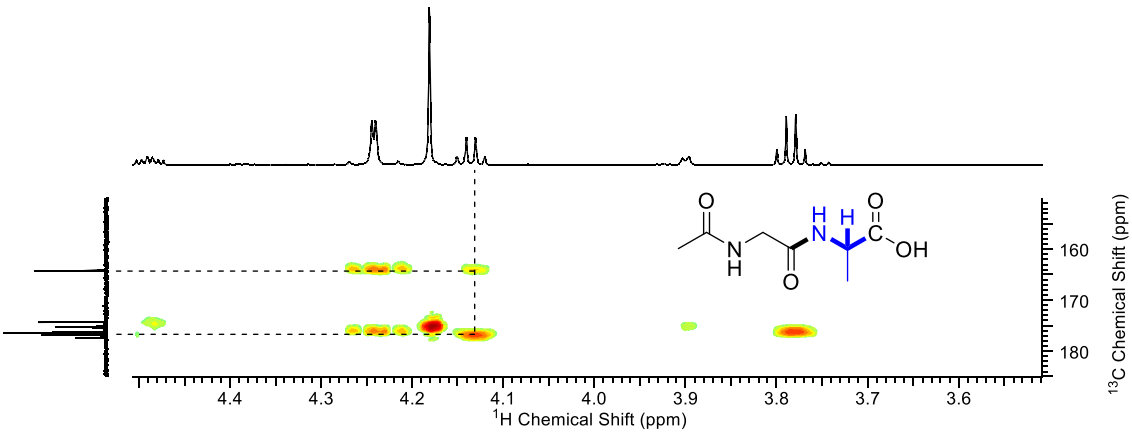


Fig. S91. ^{1}H–^{13}C HMBC (^{1}H: 700 MHz [3.5–4.5 ppm], ^{13}C: 176 MHz [150-185 ppm], H_2O/D_2O 9:1) spectrum showing the diagnostic $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling of **Ala-**αH-COOH in **Ac-Gly^{N}-Ala-OH** at 4.13 ppm with two resonances at 177 and 164 ppm, which is characteristic of amidine bond formation of DL-**Ala**. See Fig. S90 for expanded and labelled ^{1}H NMR spectrum. Stereochemistry on embedded structure is omitted for clarity.

*Coupling of N-acetylglycine nitrile **Ac-Gly-CN** with L-arginine **Arg** at pH 7 and 60 °C*

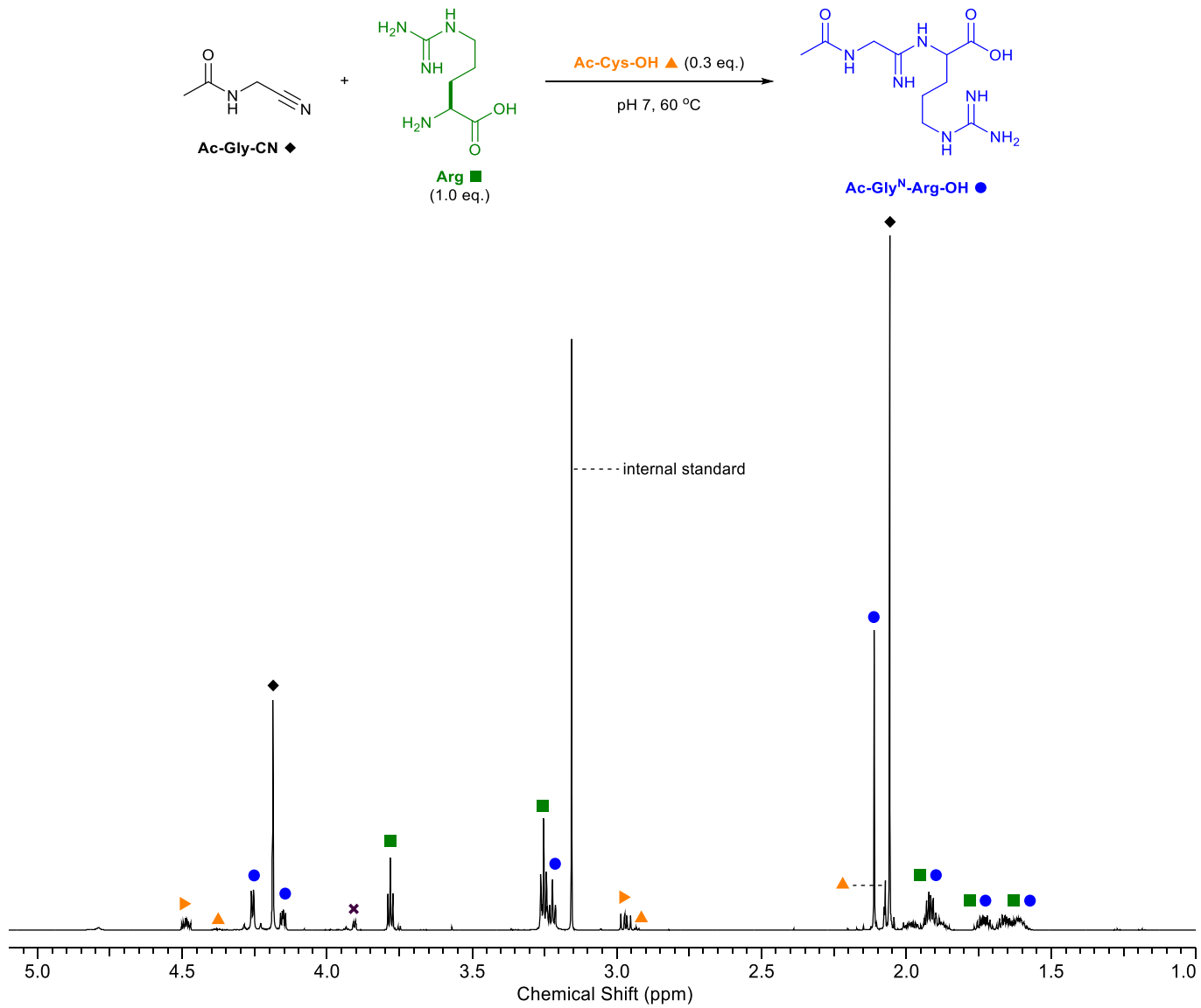


Fig. S92. ^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d, 1.0-5.0 ppm) spectrum to show the reaction of *N*-acetyl glycine nitrile (**Ac-Gly-CN**, 200 mM) with L-arginine (**Arg**, 200 mM) and *N*-acetyl-L-cysteine (**Ac-Cys-OH**, 60 mM, 0.3 eq.) with MSM (50 mM) as the internal standard after 24 h at pH 7 and 60 °C. ▶ = *N,N'*-diacetyl-L-cystine (formed by aerial oxidation of **Ac-Cys-OH**) and ✖= *N*-acetylglycinamide **Ac-Gly-NH$_2$**.

^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d) *(2-acetamido-1-iminoethyl)arginine*, **Ac-Gly^N-Arg-OH** (●) (partial assignment): δ_H 4.27 (1H, AB, *J* = 17.3 Hz, AcNHCH*H*), 4.24 (1H, AB, *J* = 17.3 Hz, AcNHCH*H*), 4.15 (1H, dd, *J* = 7.4, 4.7 Hz, Arg-αH-COOH), 3.22 (1H, t, *J* = 7.0 Hz, C*H*$_2$(guanidyl)), 2.01 (3H, s, *H*$_3$C(CO)); *L-arginine*, **Arg** (■) (partial assignment): δ_H 3.78 (1H, t, *J* = 6.2 Hz, αH-COOH), 3.25 (2H, t, *J* = 7.0 Hz, C*H*$_2$(guanidyl)); *N-Acetylglycinamide*, **Ac-Gly-NH$_2$** (✖) (partial assignment): δ_H 3.91 (2H, app. d., C*H*$_2$).

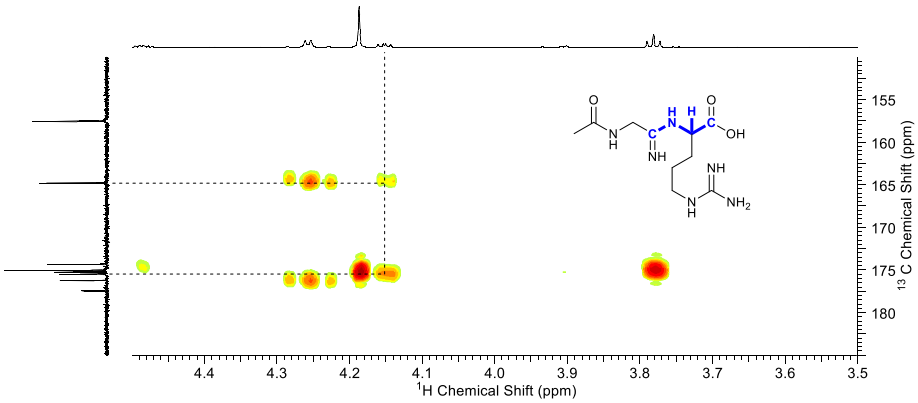


Fig. S93. ^{1}H–^{13}C HMBC (^{1}H: 700 MHz [3.5-4.5 ppm], ^{13}C: 176 MHz [150-185 ppm], H_2O/D_2O 9:1) spectrum showing the diagnostic $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling of **Arg**-αH-COOH in **Ac-Gly^{N}-Arg-OH** at 4.15 ppm with two resonances at 175 and 165 ppm, which is characteristic of amidine bond formation of **Arg**. See Fig. S92 for expanded and labelled ^{1}H NMR spectrum.

Coupling of N-acetylglycine nitrile ***Ac-Gly-CN*** *with L-asparagine* ***Asn*** *at pH 7 and 60 °C*

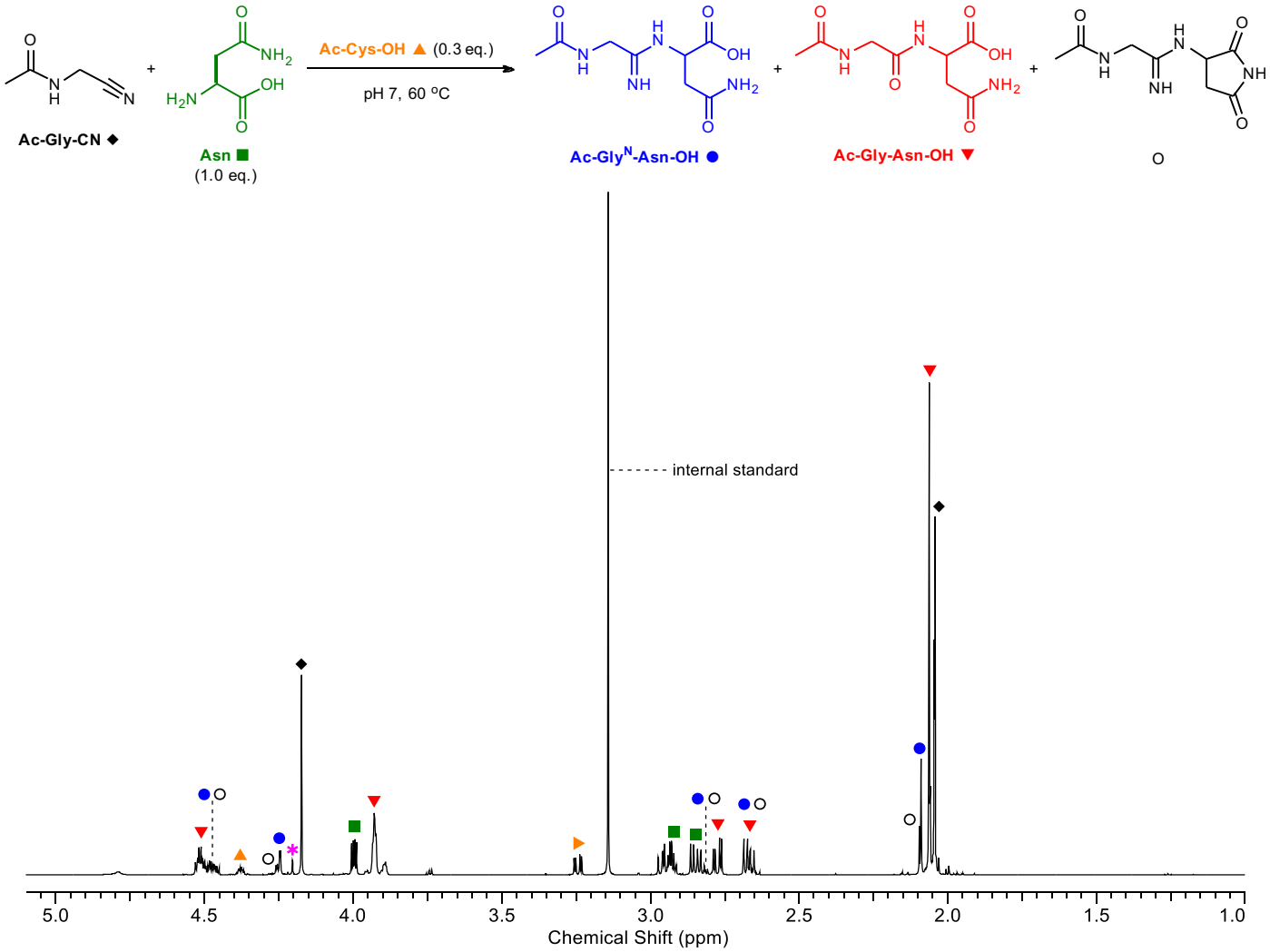


Fig. S94. ^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d, 1.0-5.0 ppm) spectrum to show the reaction of *N*-acetyl glycine nitrile (**Ac-Gly-CN**, 200 mM) with L-asparagine (**Asn**, 200 mM) and *N*-acetyl-L-cysteine (**Ac-Cys-OH**, 60 mM, 0.3 eq.) with MSM (50 mM) as the internal standard after 24 h at pH 7 and 60 °C. ▶ = *N,N'*-diacetyl-L-cystine (formed by aerial oxidation of **Ac-Cys-OH**) and ✱= *N-(2-amino-2-iminoethyl)acetamide*, **Ac-Gly^N-NH$_2$**.

^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d) *(2-acetamido-1-iminoethyl)asparagine*, **Ac-Gly^N-Asn-OH** (●) (partial assignment): δ_H 4.50-4.45 (1H, m, Asn-αH-COOH), 4.26 (1H, AB, *J* = 17.3 Hz, AcNHCH*H*), 4.23 (1H, AB, *J* = 17.5 Hz, AcNHCH*H*), 2.09 (3H, s, H_3C(CO)); *N-Acetylglycyl-L-asparagine*, **Ac-Gly-Asn-OH** (▼): δ_H 4.51 (1H, m, Asn-αH-COOH), 3.95 (1H, AB br., *J* = 17.3 Hz, AcNHCH*H*), 3.91 (1H, AB br., *J* = 18.2 Hz, AcNHCH*H*), 2.77 (1H, ABX, *J* = 15.1, 4.8 Hz, CH(C*H*HCONH$_2$)), 2.67 (1H, ABX, *J* = 15.1, 8.4 Hz, CH(C*H*HCONH$_2$)), 2.06 (3H, s, H_3C(CO)); *N-(2-((2,5-dioxopyrrolidin-3-yl)amino)-2-iminoethyl)acetamide* (O) (partial assignment): δ_H 4.50-4.45 (1H, m, C*H*(CH$_2$CONHCO)), 4.27 (1H, AB, *J* = 17.3 Hz, AcNHCH*H*), 4.24 (1H, AB, *J* = 17.3 Hz, AcNHCH*H*), 2.10 (3H, s, H_3C(CO)); *L-asparagine*, **Asn** (■): δ_H 4.00 (1H, dd, *J* = 7.6, 4.3 Hz, αH-COOH), 2.94 (1H, ABX, *J* = 17.1, 4.3 Hz, CH(C*H*HCONH$_2$)), 2.85 (1H, ABX, *J* = 16.8, 7.9 Hz, CH(C*H*HCONH$_2$)); *N-(2-amino-2-iminoethyl)acetamide*, **Ac-Gly^N-NH$_2$** (✱) (partial assignment): δ_H 4.20 (2H, s, CH_2).

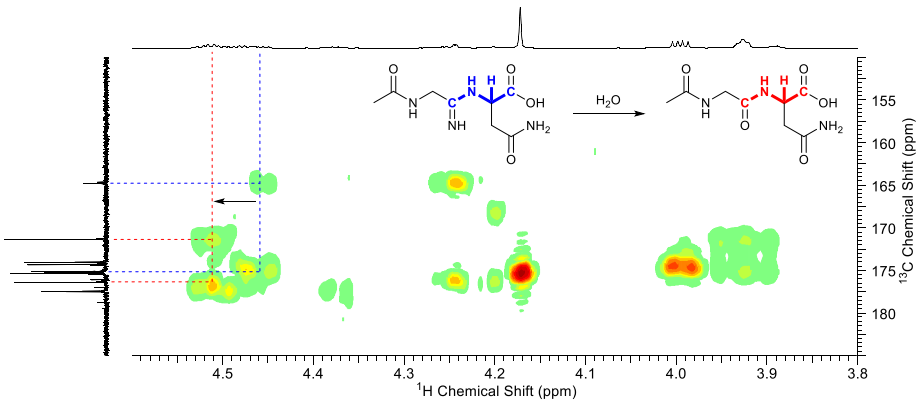
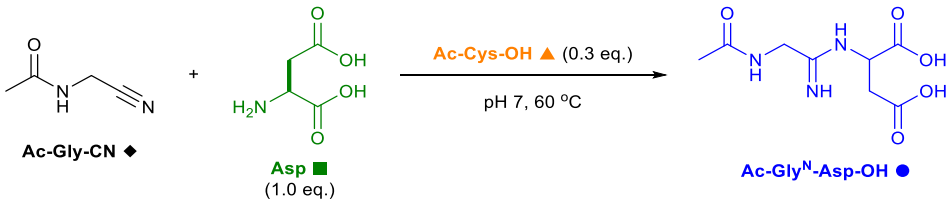


Fig. S95. ^{1}H–^{13}C HMBC (^{1}H: 700 MHz [3.8-4.8 ppm], ^{13}C: 176 MHz [150-185 ppm], H_2O/D_2O 9:1) spectrum showing the diagnostic $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling of **Asn**-αH-COOH in **Ac-Gly^{N}-Asn-OH** between 4.50-4.45 ppm with two resonances at 175 and 165 ppm, which is characteristic of amidine bond formation of **Asn**, and the $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling of **Asn**-αH-COOH in **Ac-Gly-Asn-OH** at 4.51 ppm with two resonances at 176 and 171 ppm, which is characteristic of peptide bond formation of **Asn**. See Fig. S94 for expanded and labelled ^{1}H NMR spectrum.

Coupling of N-acetylglycine nitrile ***Ac-Gly-CN*** *with L-aspartic acid* ***Asp*** *at pH 7 and 60 °C*



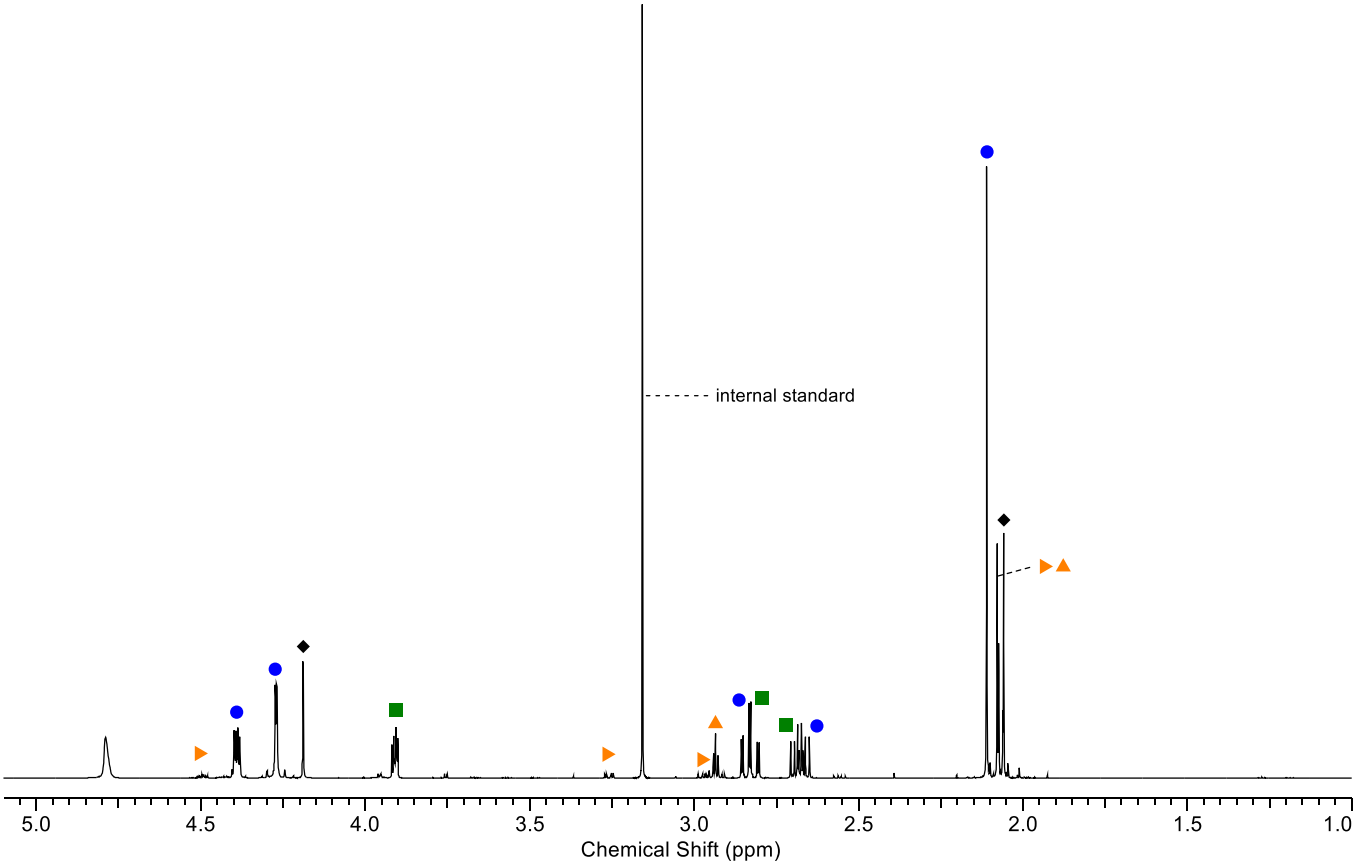


Fig. S96. ^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d, 1.0-5.0 ppm) spectrum to show the reaction of *N*-acetyl glycine nitrile (**Ac-Gly-CN**, 200 mM) with L-aspartic acid (**Asp**, 200 mM) and *N*-acetyl-L-cysteine (**Ac-Cys-OH**, 60 mM, 0.3 eq.) with MSM (50 mM) as the internal standard after 24 h at pH 7 and 60 °C. ▶ = *N,N'*-diacetyl-L-cystine (formed by aerial oxidation of **Ac-Cys-OH**) and ✖ = *N*-acetylglycinamide, **Ac-Gly-NH$_2$**.

^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d) *(2-acetamido-1-iminoethyl)aspartic acid*, **Ac-Gly^{N}-Asp-OH** (●): δ_H 4.39 (1H, dd, *J* = 8.4, 3.7 Hz, Asp-αH-COOH), 4.29 (1H, AB, *J* = 17.3 Hz, AcNHCH*H*), 4.26 (1H, AB, *J* = 17.3 Hz, AcNHCH*H*), 2.84 (1H, ABX, *J* = 16.5, 3.7 Hz, CH(CH*H*COOH)COOH), 2.67 (1H, ABX, *J* = 16.5, 8.4 Hz, CH(CH*H*COOH)COOH), 2.11 (3H, s, H_3C(CO)); *L-aspartic acid*, **Asp** (■): δ_H 3.91 (1H, dd, *J* = 8.8, 3.6 Hz, αH-COOH), 2.82 (1H, ABX, *J* = 17.5, 3.6 Hz, CH(CH*H*COOH)COOH), 2.69 (1H, ABX, *J* = 17.5, 8.8 Hz, CH(CH*H*COOH)COOH).

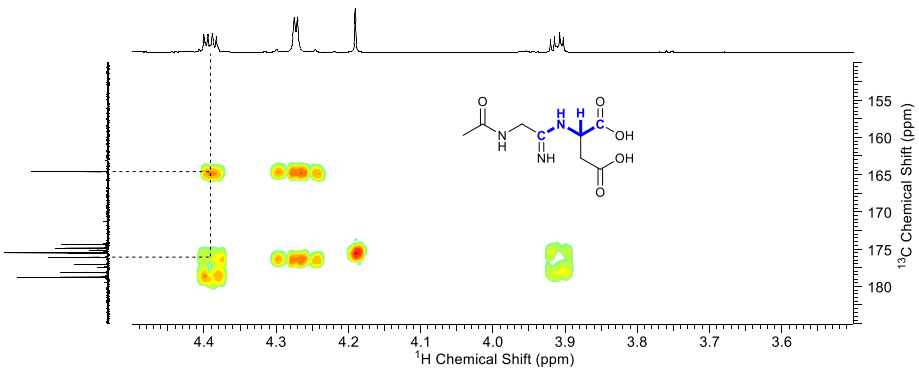
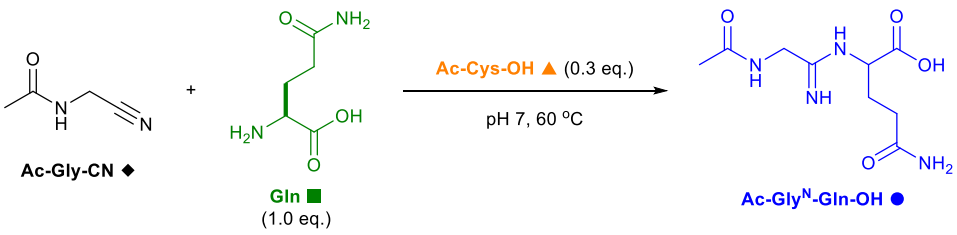


Fig. S97. ^{1}H–^{13}C HMBC (^{1}H: 700 MHz [3.5-4.5 ppm], ^{13}C: 176 MHz [150-185 ppm], H_2O/D_2O 9:1) spectrum showing the diagnostic $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling of the **Asp**-αH-COOH in **Ac-Gly^{N}-Asp-OH** at 4.39 ppm with two resonances at 176 and 165 ppm, which is characteristic of amidine bond formation of **Asp**. See Fig. S96 for expanded and labelled ^{1}H NMR spectrum.

Coupling of N-acetylglycine nitrile ***Ac-Gly-CN*** *with L-glutamine* ***Gln*** *at pH 7 and 60 °C*



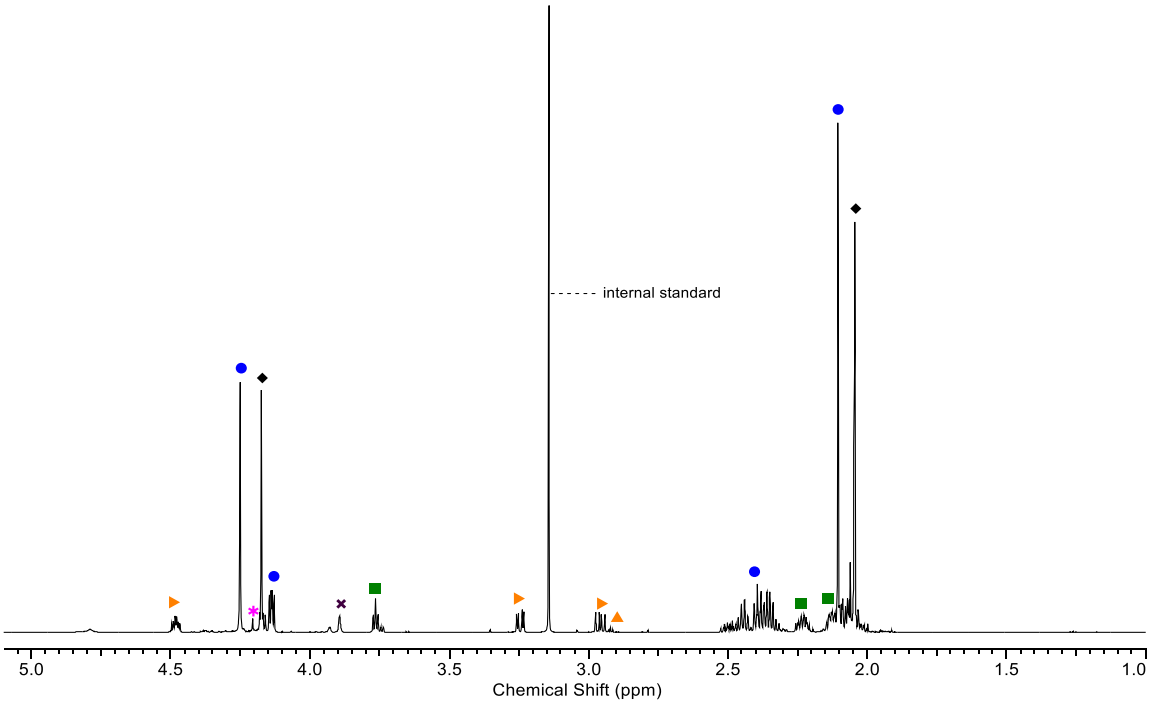


Fig. S98. ^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d, 1.0-5.0 ppm) spectrum to show the reaction of *N*-acetyl glycine nitrile (**Ac-Gly-CN**, 200 mM) with L-glutamine (**Gln**, 200 mM) and *N*-acetyl-L-cysteine (**Ac-Cys-OH**, 60 mM, 0.3 eq.) with MSM (50 mM) as the internal standard after 24 h at pH 7 and 60 °C. ▶ = *N,N'*-diacetyl-L-cystine (formed by aerial oxidation of **Ac-Cys-OH**), ✖= **Ac-Gly-NH$_2$** and ✱= **Ac-Gly^N-NH$_2$**.

^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d) *(2-acetamido-1-iminoethyl)glutamine*, **Ac-Gly^N-Gln-OH** (●): δ_H 4.25 (2H, s, AcNHCH_2), 4.14 (1H, dd, *J* = 7.5, 4.8 Hz, Gln-αH-COOH), 2.53-2.29 (4H, m, CH_2CH_2CONH$_2$), 2.10 (3H, s, H_3C(CO)); *L-glutamine*, **Gln** (■): δ_H 3.76 (1H, t, *J* = 6.2 Hz, αH-COOH 2.26-2.21 (2H, m, CH_2CH$_2$CONH$_2$), 2.14-2.00 (2H, m, CH$_2$CH_2CONH$_2$); *N-Acetylglycinamide*, **Ac-Gly-NH$_2$** (✖) (partial assignment): δ_H 3.89 (2H, s, CH_2); *N-(2-amino-2-iminoethyl)acetamide*, **Ac-Gly^N-NH$_2$** (✱) (partial assignment): δ_H 4.20 (2H, s, CH_2).

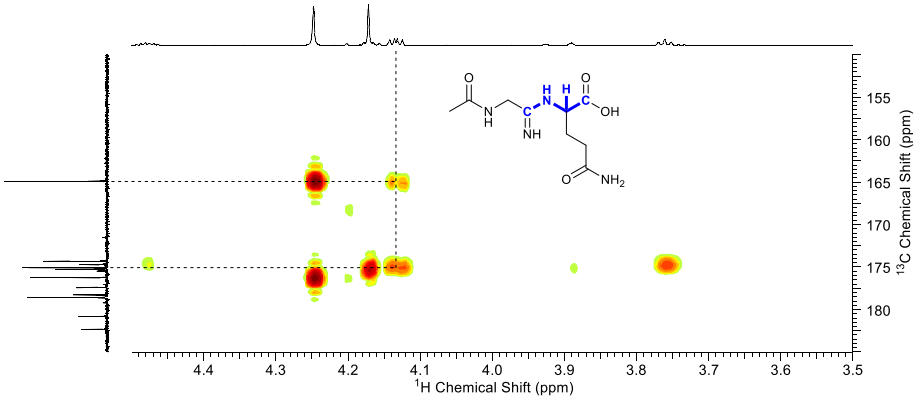
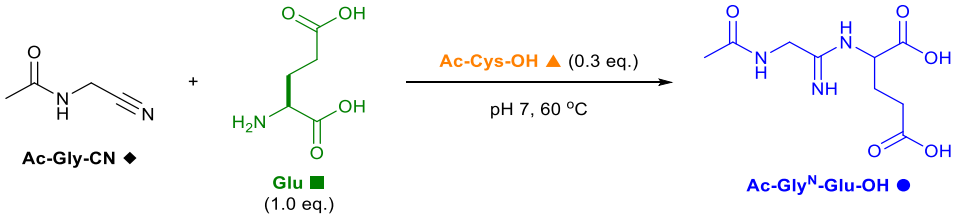


Fig. S99. ^{1}H–^{13}C HMBC (^{1}H: 700 MHz [3.5-4.5 ppm], ^{13}C: 176 MHz [150-185 ppm], H_2O/D_2O 9:1) spectrum showing the diagnostic $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling of the **Gln**-αH-COOH in **Ac-Gly^N-Gln-OH** at 4.14 ppm with two resonances at 175 and 165 ppm, which is characteristic of amidine bond formation of **Gln**. See Fig. S98 for expanded and labelled ^{1}H NMR spectrum.

Coupling of N-acetylglycine nitrile ***Ac-Gly-CN*** *with L-glutamic acid* ***Glu*** *at pH 7 and 60 °C*



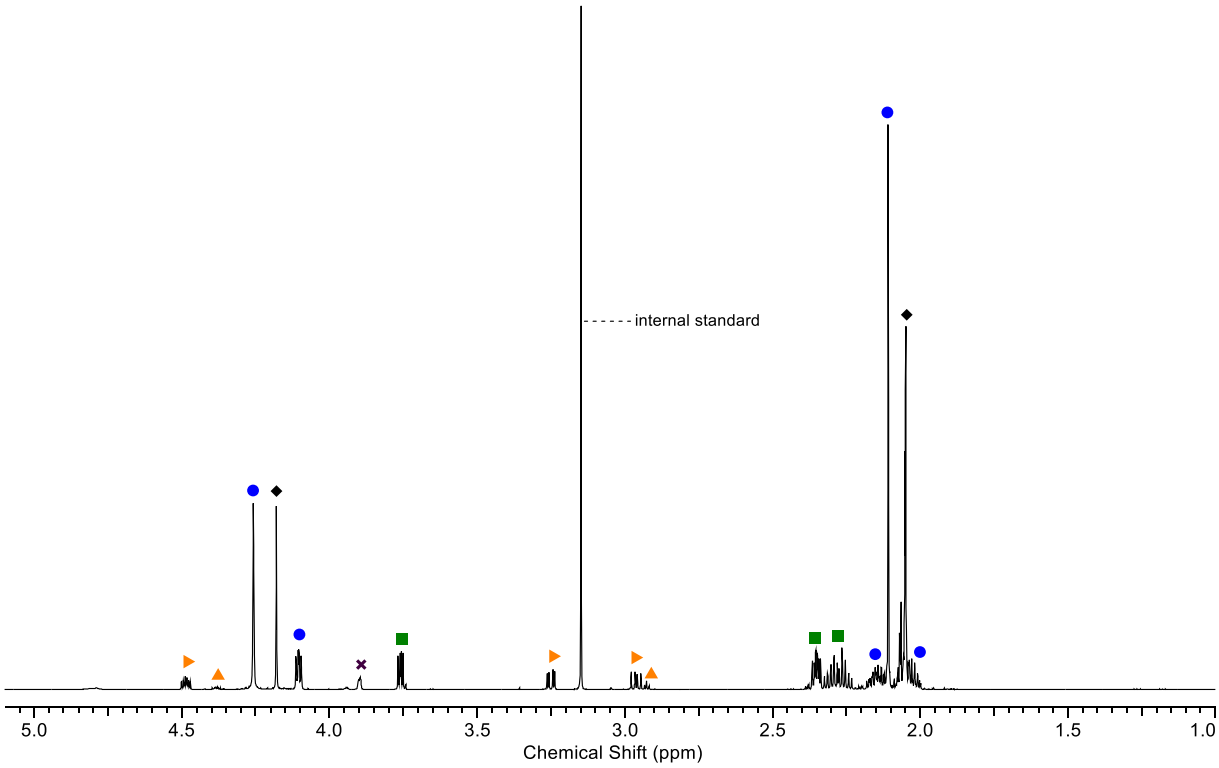


Fig. S100. ^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d, 1.0-5.0 ppm) spectrum to show the reaction of *N*-acetyl glycine nitrile (**Ac-Gly-CN**, 200 mM) with L-glutamic acid (**Glu**, 200 mM) and *N*-acetyl-L-cysteine (**Ac-Cys-OH**, 60 mM, 0.3 eq.) with MSM (50 mM) as the internal standard after 24 h at pH 7 and 60 °C. ▶ = *N,N'*-diacetyl-L-cystine (formed by aerial oxidation of **Ac-Cys-OH**) and ✖= **Ac-Gly-NH$_2$**.

^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d) *(2-acetamido-1-iminoethyl)glutamic acid,* **Ac-Gly^{N}-Glu-OH** (●): δ_H δ4.26 (2H, s, AcNHC*H*$_2$), 4.10 (1H, dd, *J* = 7.4, 5.2 Hz, Glu-αH-COOH), 2.18-2.10 (2H, m, CH_2CH_2COOH), 2.11 (3H, s, $H_3C(CO)$), 2.08-2.00 (2H, m, CH_2CH_2COOH); *L-glutamic acid*, **Glu** (■): δ_H 3.76 (1H, dd, *J* = 7.2, 4.7 Hz, αH-COOH), 2.39-2.34 (2H, m, CH_2CH_2COOH), 2.32-2.23 (2H, m, CH_2CH_2COOH); *N-Acetylglycinamide*, **Ac-Gly-NH$_2$** (✖) (partial assignment): δ_H 3.90 (2H, AB obs., C*H*$_2$).

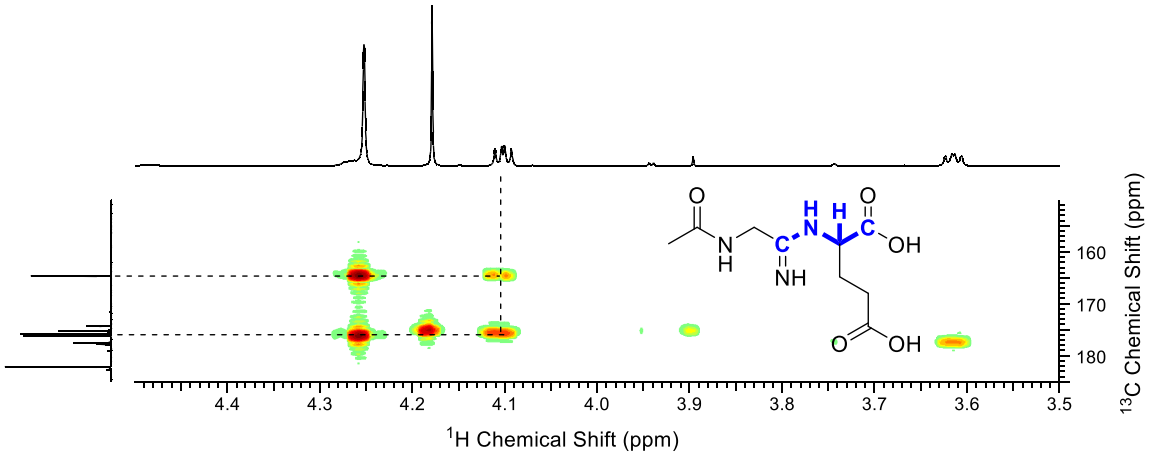


Fig. S101. ^{1}H–^{13}C HMBC (^{1}H: 700 MHz [3.5-4.5 ppm], ^{13}C: 176 MHz [150-185 ppm], H_2O/D_2O 9:1) spectrum showing the diagnostic $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling of the **Glu**-αH-COOH in **Ac-Gly^{N}-Glu-OH** at 4.10 ppm with two resonances at 176 and 165 ppm, which is characteristic of amidine bond formation of **Glu**. See Fig. S100 for expanded and labelled ^{1}H NMR spectrum.

*Coupling of N-acetylglycine nitrile **Ac-Gly-CN** with L-histidine **His** at pH 7 and 60 °C*

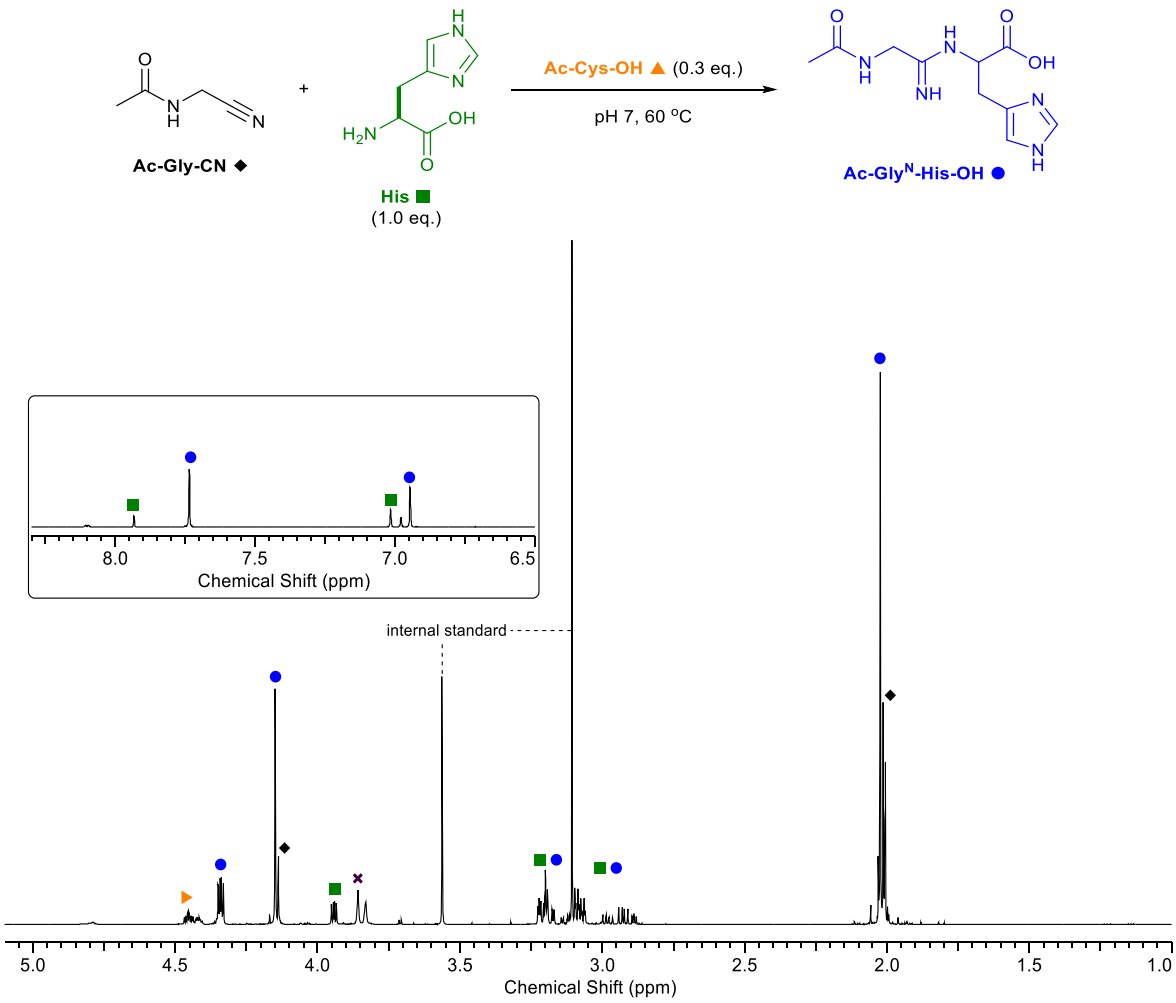


Fig. S102. ^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d, 1.0-5.0 ppm) spectrum to show the reaction of *N*-acetyl glycine nitrile (**Ac-Gly-CN**, 200 mM) with L-histidine (**His**, 200 mM) and *N*-acetyl-L-cysteine (**Ac-Cys-OH**, 60 mM, 0.3 eq.) with MSM (50 mM) and pentaerythritol (14.29 mM) as the internal standard after 24 h at pH 7 and 60 °C. Inset: ^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d, 6.5-8.3 ppm) showing the aromatic CH resonances present in **His** and **Ac-Gly^{N}-His-OH**. ▶ = *N,N'*-diacetyl-L-cystine (formed by aerial oxidation of **Ac-Cys-OH**) and ✖= **Ac-Gly-NH$_2$**.

^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d) *(2-Acetamido-1-iminoethyl)histidine*, **Ac-Gly^{N}-His-OH** (●) (partial assignment): δ_H 7.74 (1H, s, Ar*H*), 6.95 (1H, s, Ar*H*), 4.34 (1H, dd, *J* = 7.9, 4.7 Hz, His-αH-COOH), 4.15 (2H, s, AcNHC*H*$_2$), 2.02 (3H, s, *H*$_3$C(CO)); *L-Histidine*, **His** (■) (partial assignment): δ_H 7.93 (1H, d, *J* = 0.9 Hz, Ar*H*), 7.01 (1H, d, *J* = 0.9 Hz, Ar*H*), 3.94 (1H, dd, *J* = 8.1, 4.7 Hz, αH-COOH); *N-Acetylglycinamide*, **Ac-Gly-NH$_2$** (✖) (partial assignment): δ_H 3.86 (2H, s br., C*H*$_2$).

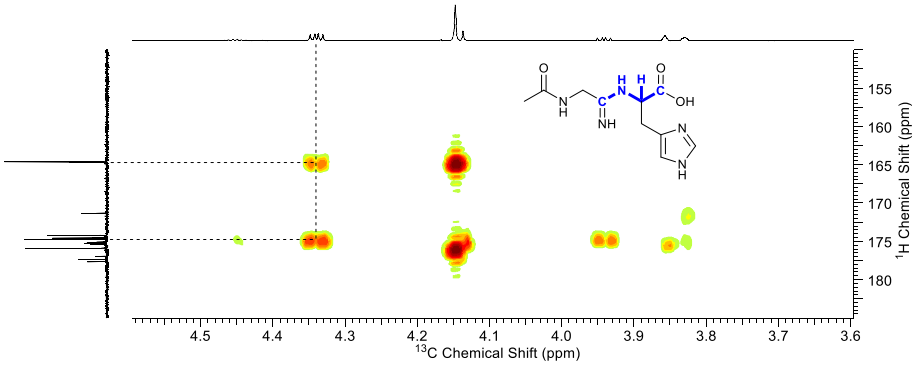


Fig. S103. ^{1}H–^{13}C HMBC (^{1}H: 700 MHz [3.6-4.6 ppm], ^{13}C: 176 MHz [150-185 ppm], H_2O/D_2O 9:1) spectrum showing the diagnostic $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling of the **His**-αH-COOH in **Ac-Gly^{N}-His-OH** at 4.34 ppm with two resonances at 175 and 165 ppm, which is characteristic of amidine bond formation of **His**. See Fig. S102 for expanded and labelled ^{1}H NMR spectrum.

*Coupling of N-acetylglycine nitrile **Ac-Gly-CN** with L-isoleucine **Ile** at pH 7 and 60 °C*

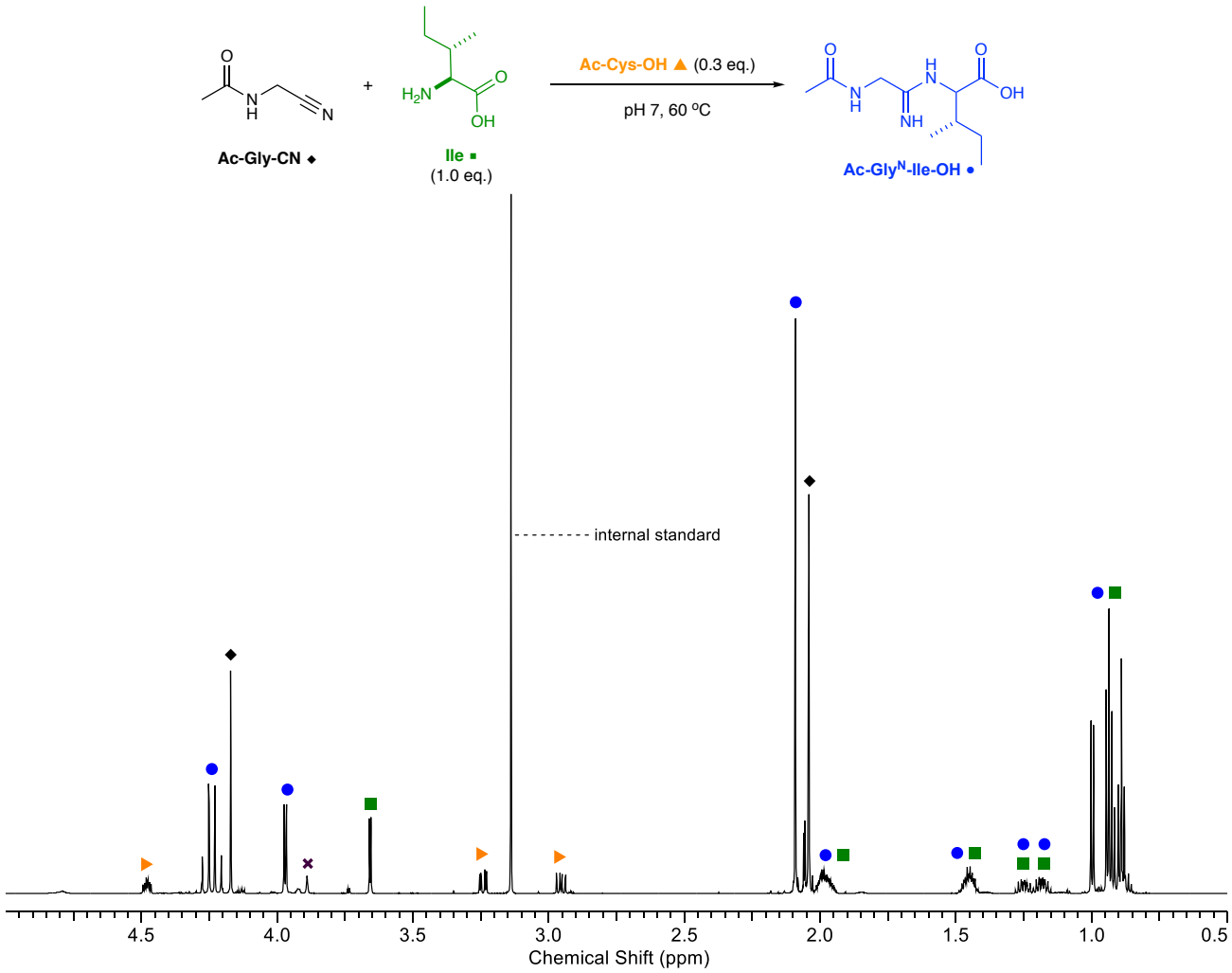


Fig. S104. 1H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d, 0.5–5.0 ppm) spectrum to show the reaction of *N*-acetyl glycine nitrile (**Ac-Gly-CN**, 200 mM) with L-isoleucine (**Ile**, 200 mM) and *N*-acetyl-L-cysteine (**Ac-Cys-OH**, 60 mM, 0.3 eq.) with MSM (50 mM) as the internal standard after 24 h at pH 7 and 60 °C. ▶ = *N,N'*-diacetyl-L-cystine (formed by aerial oxidation of **Ac-Cys-OH**) and ✖ = **Ac-Gly-NH$_2$**.

^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d) *(2-acetamido-1-iminoethyl)isoleucine*, **Ac-Gly^N-Ile-OH** (●) (partial assignment): δ_H 4.26 (1H, AB, *J* = 17.1 Hz, AcNHCH*H*), 4.22 (1H, AB, *J* = 17.1 Hz, AcNHCH*H*), 3.97 (1H, d, *J* = 5.8 Hz, Ile-αH-COOH); *L-isoleucine*, **Ile** (■) (partial assignment): δ_H 3.66 (1H, d, *J* = 3.8 Hz, αH-COOH); *N-Acetylglycinamide*, **Ac-Gly-NH$_2$** (✖) (partial assignment): δ_H 3.89 (2H, s, C*H*$_2$).

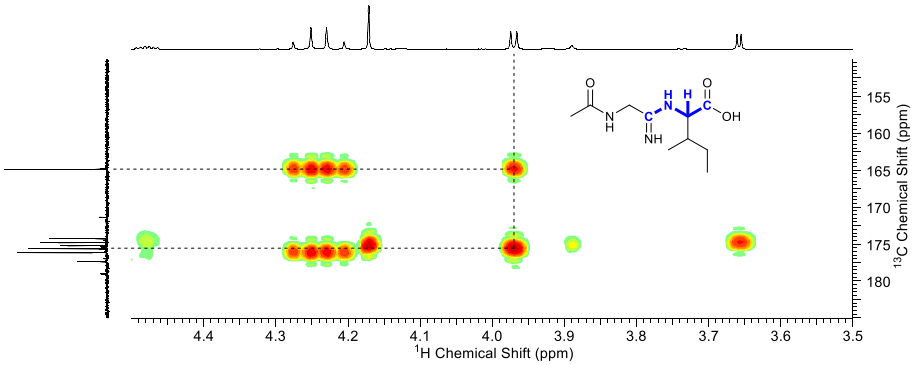


Fig. S105. ^{1}H–^{13}C HMBC (^{1}H: 700 MHz [3.5–4.5 ppm], ^{13}C: 176 MHz [150-185 ppm], H_2O/D_2O 9:1) spectrum showing the diagnostic $^2J_{CH}$ and $^3J_{CH}$ coupling of the **Ile**-αH-COOH in **Ac-Gly^N-Ile-OH** at 4.26 ppm with two resonances at 176 and 165 ppm, which is characteristic of amidine bond formation of **Ile**. See Fig. S104 for expanded and labelled ^{1}H NMR spectrum.

*Coupling of N-acetylglycine nitrile **Ac-Gly-CN** with L-leucine **Leu** at pH 7 and 60 °C*

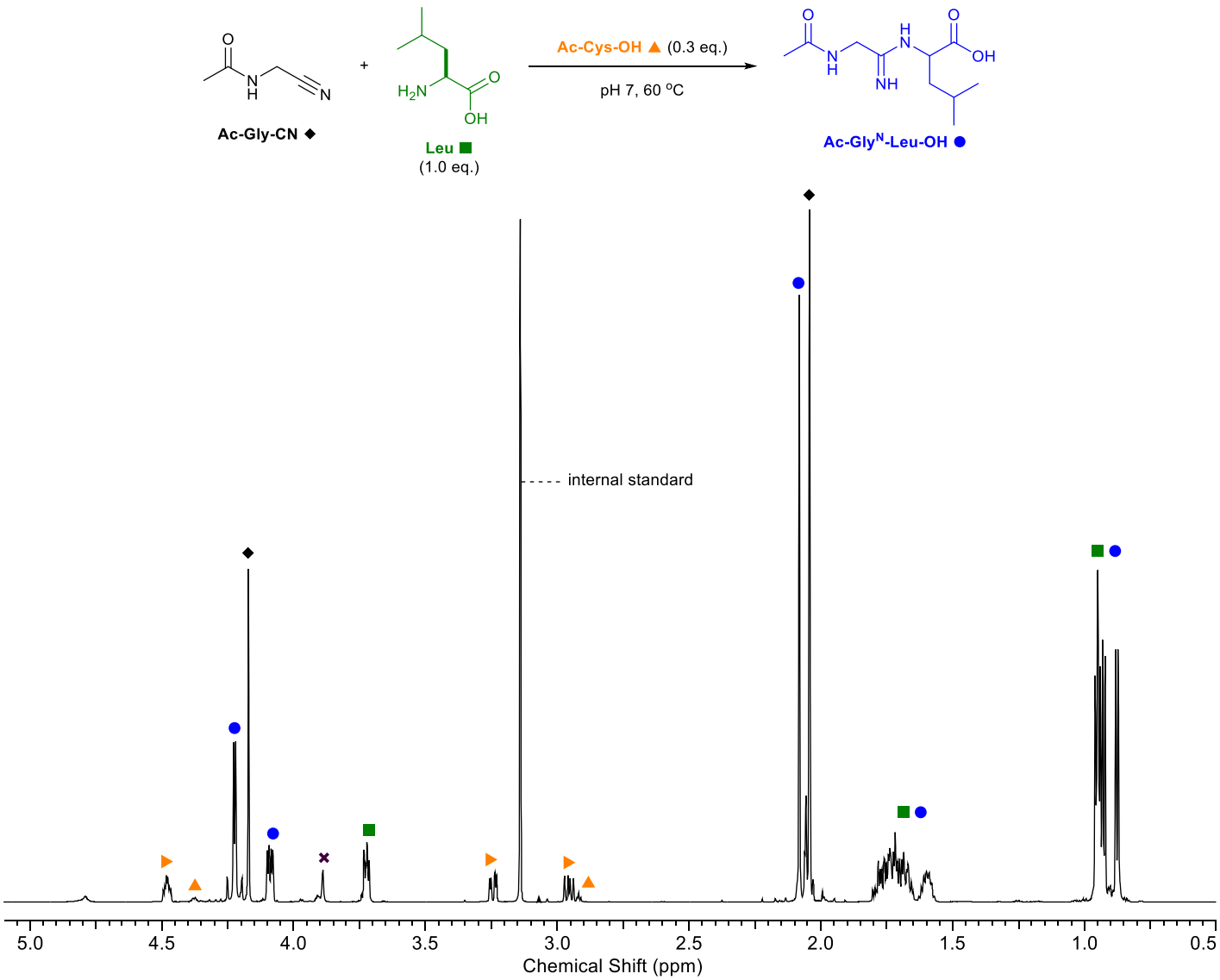


Fig. S106. ^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d, 0.5–5.0 ppm) spectrum to show the reaction of *N*-acetyl glycine nitrile (**Ac-Gly-CN**, 200 mM) with L-leucine (**Leu**, 200 mM) and *N*-acetyl-L-cysteine (**Ac-Cys-OH**, 60 mM, 0.3 eq.) with MSM (50 mM) as the internal standard after 24 h at pH 7 and 60 °C. ▶ = *N,N'*-diacetyl-L-cystine (formed by aerial oxidation of **Ac-Cys-OH**) and ✖ = **Ac-Gly-NH$_2$**.

^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d) *(2-acetamido-1-iminoethyl)leucine*, **Ac-Gly^{N}-Leu-OH** (●) (partial assignment): δ_H 4.24 (1H, AB, *J* = 17.1 Hz, AcNHCH*H*), 4.21 (1H, AB, *J* = 17.1 Hz, AcNHCH*H*), 4.09 (1H, dd, *J* = 9.6, 4.5 Hz, Leu-αH-COOH), 2.08 (3H, s, *H*$_3$C(CO)); *L-leucine*, **Leu** (■) (partial assignment): δ_H 3.72 (1H, dd, *J* = 8.3, 5.2 Hz, αH-COOH); *N-Acetylglycinamide*, **Ac-Gly-NH$_2$** (✖) (partial assignment): δ_H 3.89 (2H, s, C*H*$_2$).

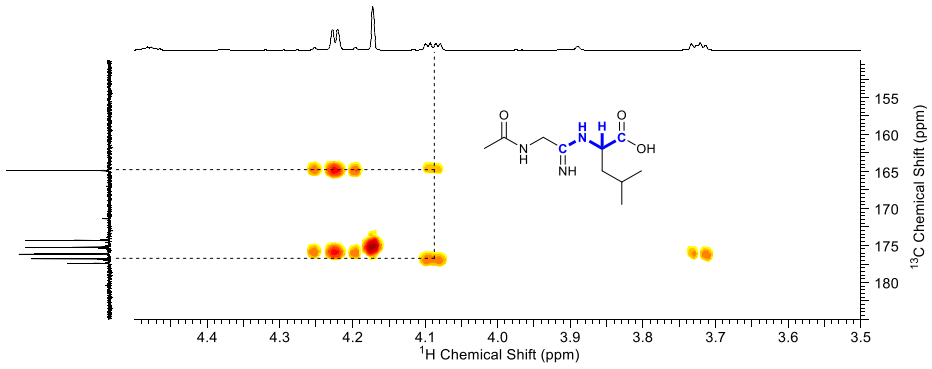
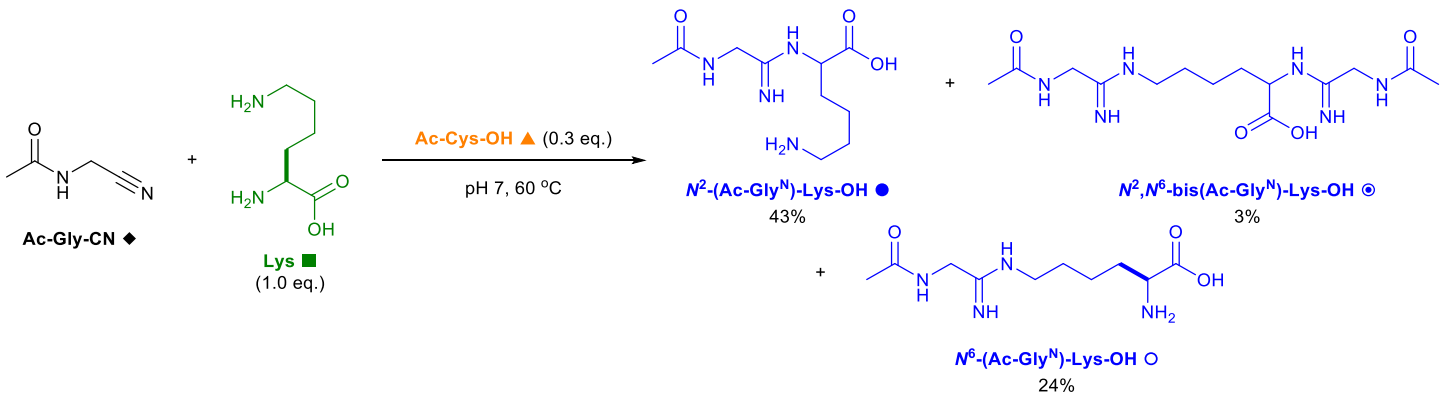


Fig. S107. ^{1}H–^{13}C HMBC (^{1}H: 700 MHz [3.5–4.5 ppm], ^{13}C: 176 MHz [150–185 ppm], H_2O/D_2O 9:1) spectrum showing the diagnostic $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling of the **Leu**-αH-COOH in **Ac-Gly^{N}-Leu-OH** at 4.09 ppm with two resonances at 178 and 165 ppm, which is characteristic of amidine bond formation of **Leu**. See Fig. S106 for expanded and labelled ^{1}H NMR spectrum.

Coupling of N-acetylglycine nitrile ***Ac-Gly-CN*** *with L-lysine* ***Lys*** *at pH 7 and 60 °C*



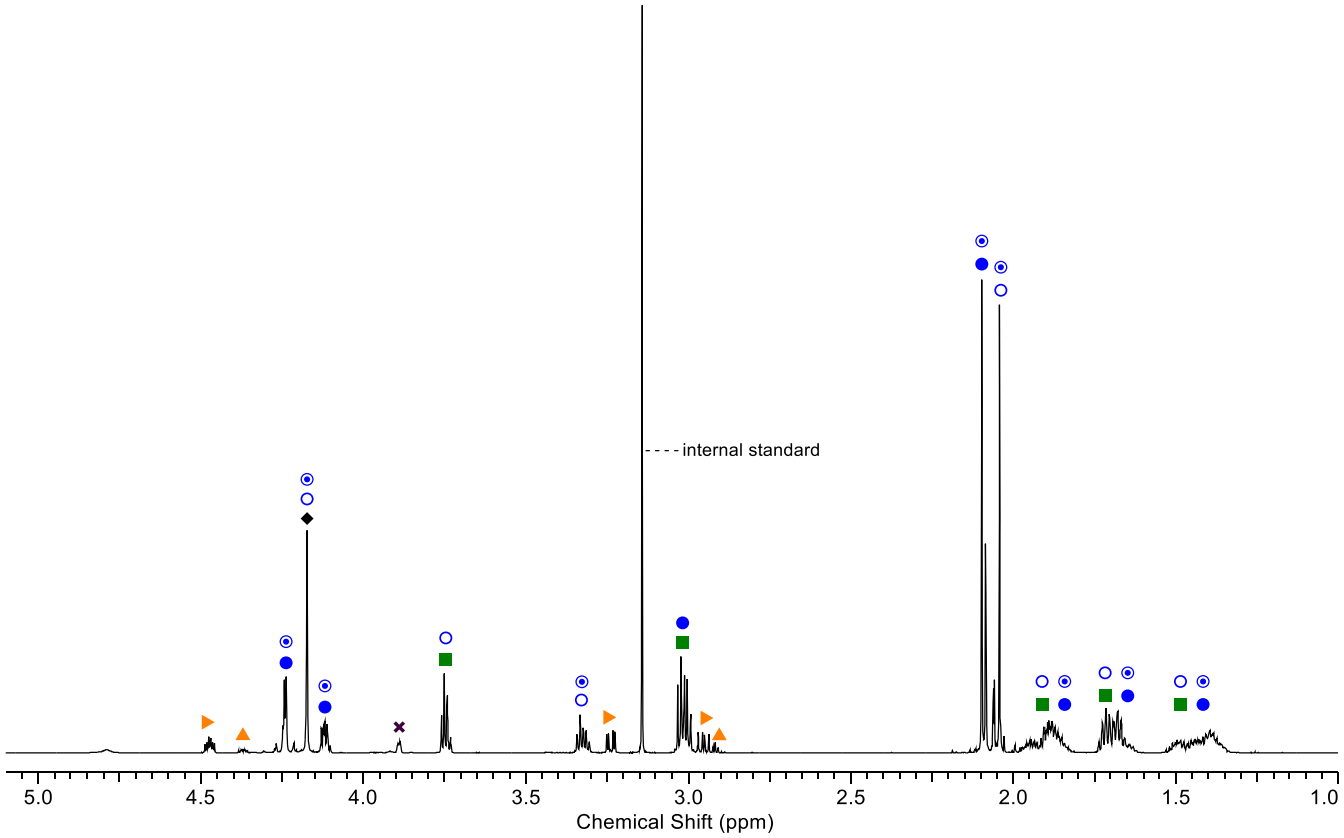


Fig. S108. ^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d, 1.0–5.0 ppm) spectrum to show the reaction of *N*-acetyl glycine nitrile (**Ac-Gly-CN**, 200 mM) with L-lysine (**Lys**, 200 mM) and *N*-acetyl-L-cysteine (**Ac-Cys-OH**, 60 mM, 0.3 eq.) with MSM (50 mM) as the internal standard after 24 h at pH 7 and 60 °C. ▶ = *N,N'*-diacetyl-L-cystine (formed by aerial oxidation of **Ac-Cys-OH**) and ✖= **Ac-Gly-NH$_2$**.

^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d) *N^2-(2-acetamido-1-iminoethyl)lysine*, **N^2-(Ac-Gly^N)-Lys-OH** (●) (partial assignment): δ_H 4.26 (1H, AB, *J* = 17.3 Hz, *N*^{2}-AcNHCH*H*), 4.24-4.22 (1H, obs., *N*^{2}-AcNHCH*H*), 4.13-4.10 (1H, m, Lys-αH-COOH), 3.02 (2H, t, *J* = 7.6 Hz, *N*^{6}-H_2NCH_2CH$_2$), 2.10 (3H, s, *N*^{2}-H_3C(CO)); *N^2,N^6-di(2-acetamido-1-iminoethyl)lysine*, **N^2,N^6-di(Ac-Gly^N)-Lys-OH** (⊙) (partial assignment): δ_H 4.26 (1H, AB, *J* = 17.1 Hz, *N*^{2}-AcNHCH*H*), 4.22 (1H, AB, *J* = 17.1 Hz, *N*^{2}-AcNHCH*H*), 4.17 (2H, s br., *N*^{6}-AcNHCH_2), 4.12 (1H, dd, *J* = 7.4, 4.7 Hz, lysyl-αH-COOH), 3.33 (2H, t, *J* = 7.0 Hz, *N*^{6}-CH_2CH$_2$), 2.10 (3H, s, *N*^{2}-H_3C(CO)), 2.04 (3H, s, *N*^{6}-H_3C(CO)); N^6-*(2-acetamido-1-iminoethyl)lysine*, **N^6-(Ac-Gly^N)-Lys-OH** (○) (partial assignment): δ_H 4.17 (2H, s br., *N*^{6}-AcNHCH_2), 3.75 (1H, t, *J* = 6.1 Hz, C*H*, Lys-αH-COOH), 3.32-3.30 (2H, m, *N*^{6}-CH_2CH$_2$), 2.04 (3H, s, *N*^{6}-H_3C(CO)); *L-lysine*, **Lys** (■) (partial assignment): δ_H 3.76-3.73 (1H, m, C*H*, Lys-αH-COOH), 3.00 (2H, t, *J* = 7.4 Hz, *N*^{6}-H_2NCH_2CH$_2$); *N-Acetylglycinamide*, **Ac-Gly-NH$_2$** (✖) (partial assignment): δ_H 3.89 (2H, app. d, *J* = 2.9 Hz, CH_2).

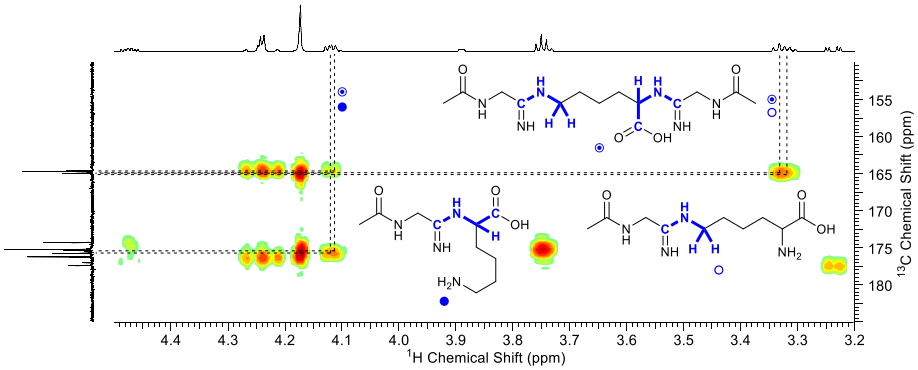


Fig. S109. ^{1}H–^{13}C HMBC (^{1}H: 700 MHz [3.2–4.5 ppm], ^{13}C: 176 MHz [150–185 ppm], H_2O/D_2O 9:1) spectrum showing the diagnostic $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling of the **lysyl**-αH-COOH and **lysyl**-N^{6}-CH_2 in ***N*^{2}-(Ac-Gly^{N})-Lys-OH** (●), ***N*^{2},*N*^{6}-di(Ac-Gly^{N})-Lys-OH** (⦿), ***N*^{6}-(Ac-Gly^{N})-Lys-OH** (○) which are characteristic of amidine bond formations of **Lys**. See Fig. S108 for expanded and labelled ^{1}H NMR spectrum.

*Coupling of N-acetylglycine nitrile **Ac-Gly-CN** with DL-methionine DL-**Met** at pH 7 and 60 °C*

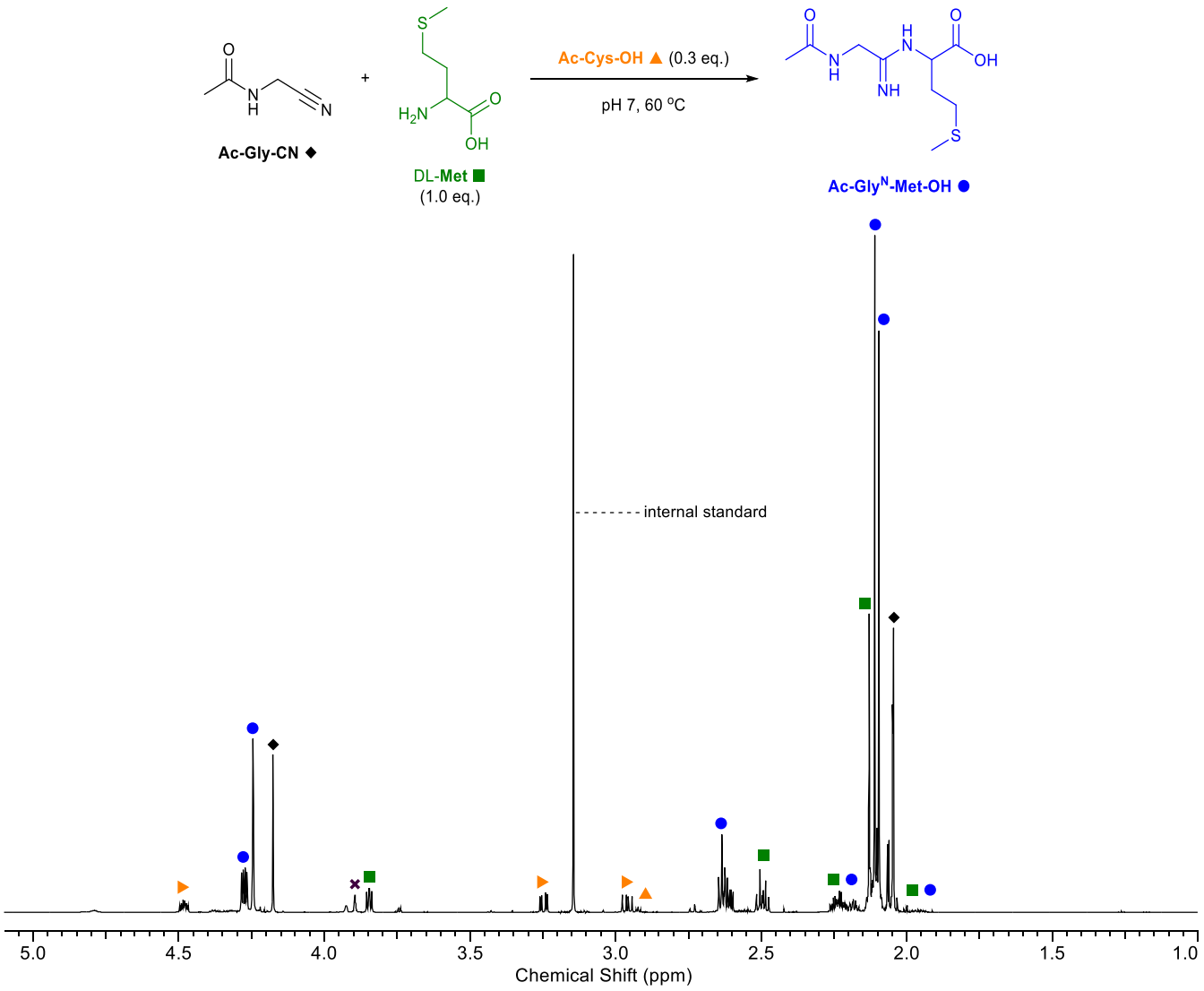


Fig. S110. ^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d, 1.0-5.0 ppm) spectrum to show the reaction of *N*-acetyl glycine nitrile (**Ac-Gly-CN**, 200 mM) with DL-methionine (DL-**Met**, 200 mM) and *N*-acetyl-L-cysteine (**Ac-Cys-OH**, 60 mM, 0.3 eq.) with MSM (50 mM) as the internal standard after 24 h at pH 7 and 60 °C. ▶ = *N,N'*-diacetyl-L-cystine (formed by aerial oxidation of **Ac-Cys-OH**) and ✖= **Ac-Gly-NH$_2$**.

^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d) *(2-acetamido-1-iminoethyl)methionine*, **Ac-Gly^{N}-Met-OH** (●): δ_H 4.27 (1H, dd, *J* = 8.5, 4.3 Hz, Met-αH-COOH), 4.24(2H, s, AcNHC*H*$_2$), 2.65-2.60 (2H, m, C*H*$_2$SCH$_3$), 2.11 (3H, s, CH$_2$SC*H*$_3$), 2.10 (3H, s, *H*$_3$C(CO)); *DL-methionine*, *DL*-**Met** (■) (partial assignment): δ_H 3.85 (1H, dd, *J* = 7.2, 5.4 Hz, αH-COOH), 2.49 (2H, m, C*H*$_2$SCH$_3$), 2.13 (3H, s, CH$_2$SC*H*$_3$); *N-Acetylglycinamide*, **Ac-Gly-NH$_2$** (✖) (partial assignment): δ_H 3.89 (2H, s, C*H*$_2$).

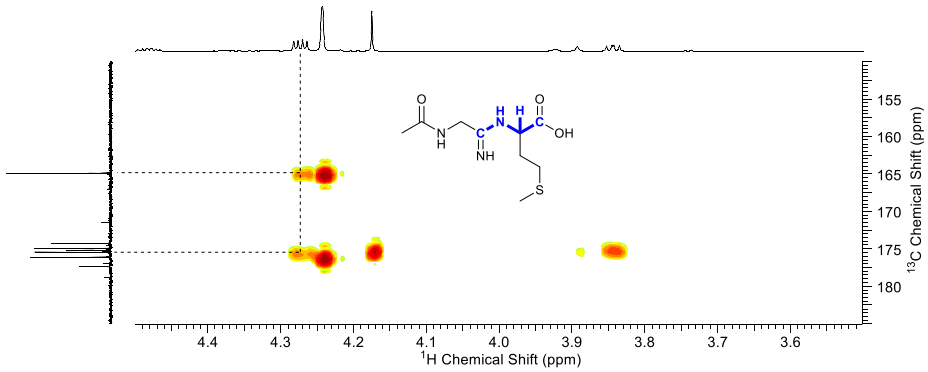


Fig. S111. ^{1}H–^{13}C HMBC (^{1}H: 700 MHz [3.5–4.5 ppm], ^{13}C: 176 MHz [150-185 ppm], H_2O/D_2O 9:1) spectrum showing the diagnostic $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling of the DL-**Met**-αH-COOH in **Ac-Gly^{N}-Met-OH** at 4.27 ppm with two resonances at 175 and 165 ppm, which is characteristic of amidine bond formation of DL-**Met**. See Fig. S110 for expanded and labelled ^{1}H NMR spectrum.

*Coupling of N-acetylglycine nitrile **Ac-Gly-CN** with L-phenylalanine **Phe** at pH 7 and 60 °C*

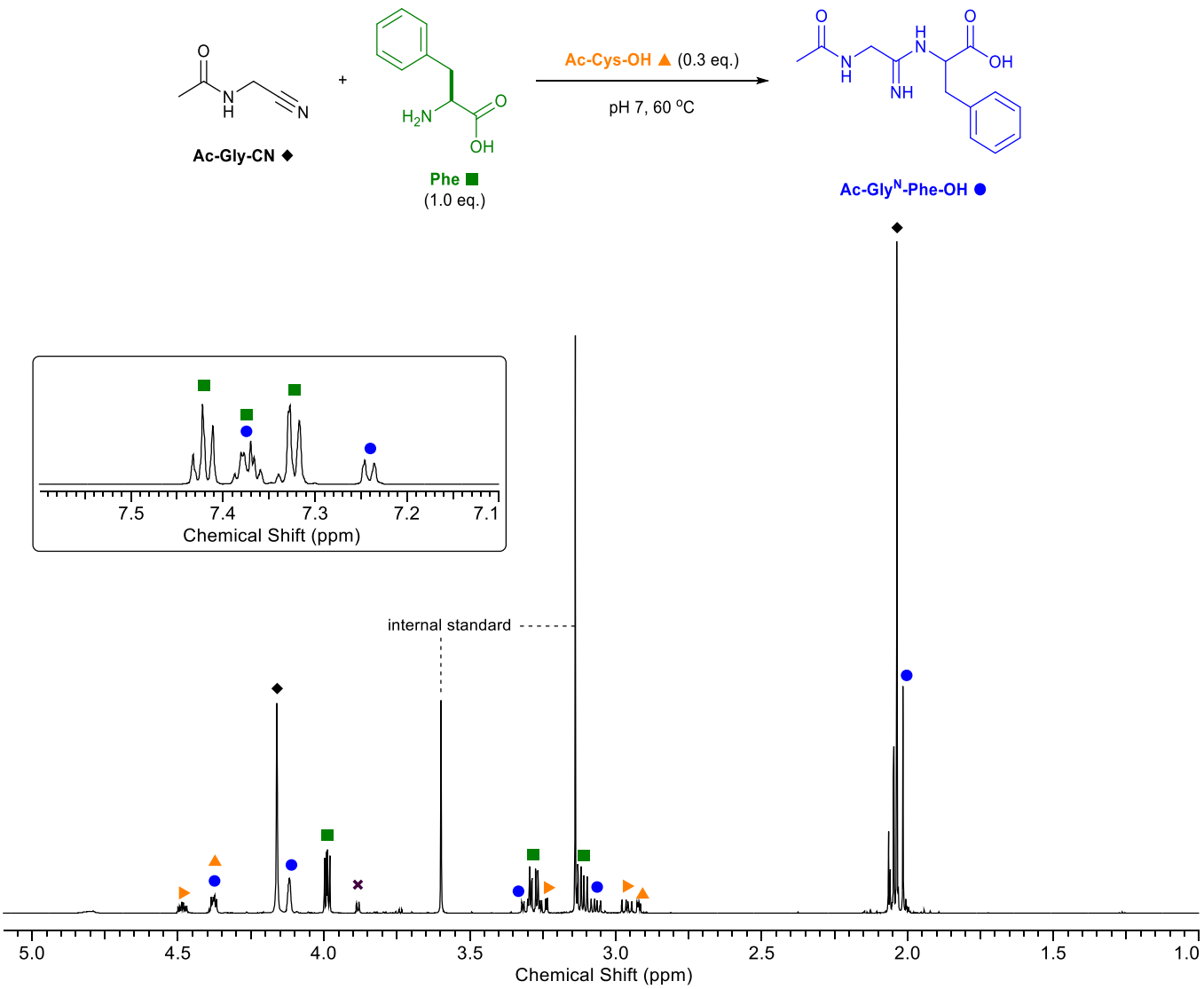


Fig. S112. ^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d, 1.0–5.0 ppm) spectrum to show the reaction of *N*-acetyl glycine nitrile (**Ac-Gly-CN**, 200 mM) with L-phenylalanine (**Phe**, 200 mM) and *N*-acetyl-L-cysteine (**Ac-Cys-OH**, 60 mM, 0.3 eq.) with MSM (50 mM) and pentaerythritol (14 mM) as internal standards after 24 h at pH 7 and 60 °C. Inset: ^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d, 7.1-7.6 ppm) showing the aromatic CH resonances present in **Phe** and **Ac-Gly^N-Phe-OH**. ▶ = *N,N′*-diacetyl-L-cystine (formed by aerial oxidation of **Ac-Cys-OH**) and ✖ = **Ac-Gly-NH$_2$**.

^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d) *(2-acetamido-1-iminoethyl)phenylalanine*, **Ac-Gly^N-Phe-OH** (●): δ_H 7.39-7.36 (3H, m, Ar*H*), 7.24 (2H, d, *J* = 7.2 Hz, Ar*H*), 4.38 (1H, ABX, *J* = 7.9, 4.7 Hz, Phe-αH-COOH), 4.12 (2H, br. s, AcNHC*H*$_2$), 3.31 (1H, dd, *J* = 14.2, 47 Hz, CHCH*H*Ph), 3.07 (1H, dd, *J* = 14.2, 7.9 Hz, CHCH*H*Ph), 2.01 (3H, s, *H*$_3$C(CO)); *L-phenylalanine*, **Phe** (■): δ_H 7.43-7.41 (2H, m, Ar*H*), 7.39-7.36 (1H, m, Ar*H*), 7.34-7.32 (2H, m, Ar*H*), 3.99 (1H, ABX, *J* = 7.9, 5.2 Hz, αH-COOH), 3.28 (1H, dd, *J* = 14.5, 5.3 Hz, CHCH*H*Ph), 3.11 (1H, dd, *J* = 14.6, 8.1 Hz, CHCH*H*Ph); *N-Acetylglycinamide*, **Ac-Gly-NH$_2$** (✖) (partial assignment): δ_H 3.88 (2H, AB obs., C*H*$_2$).

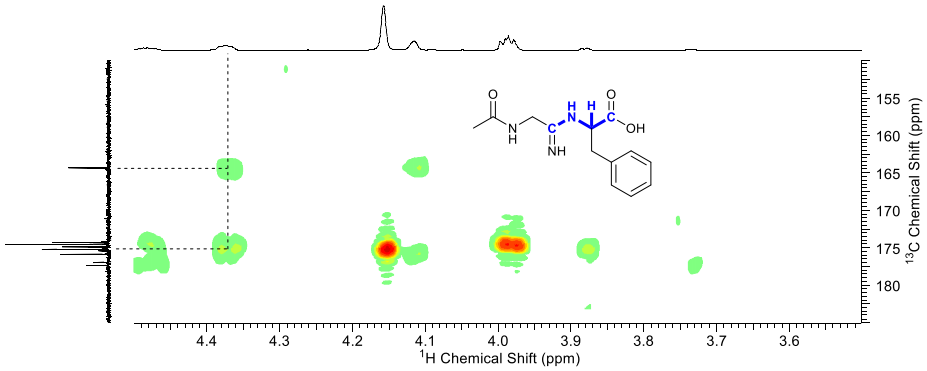


Fig. S113. ^{1}H–^{13}C HMBC (^{1}H: 700 MHz [3.5–4.5 ppm], ^{13}C: 176 MHz [150–185 ppm], H_2O/D_2O 9:1) spectrum showing the diagnostic $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling of the **Phe**-αH-COOH in **Ac-Gly^{N}-Phe-OH** at 4.38 ppm with two resonances at 175 and 164 ppm, which is characteristic of amidine bond formation of **Phe**. See Fig. S112 for expanded and labelled ^{1}H NMR spectrum.

Coupling of N-acetylglycine nitrile ***Ac-Gly-CN*** *with L-proline* ***Pro*** *at pH 7 and 60 °C*

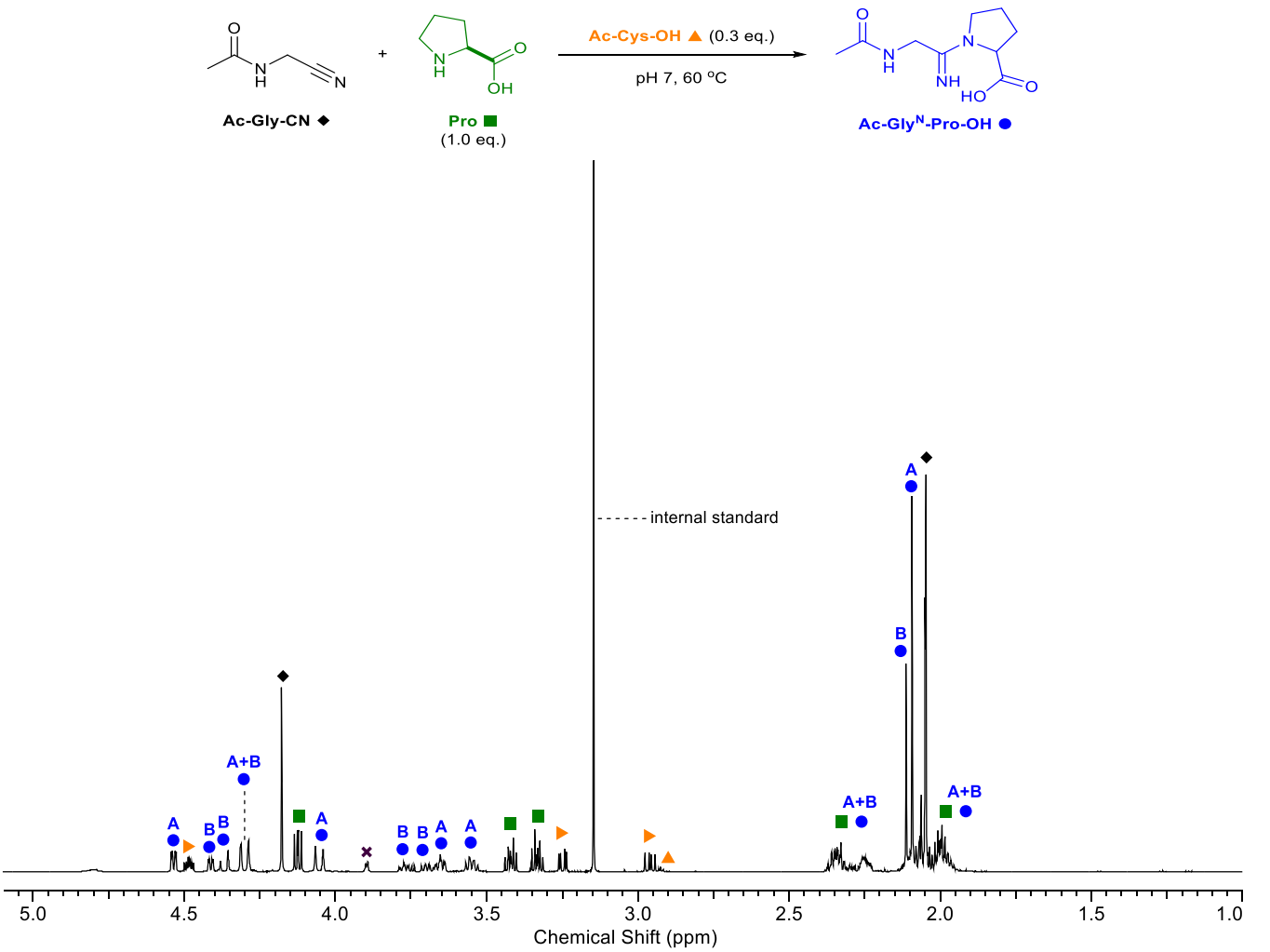


Fig. S114. ^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d, 1.0-5.0 ppm) spectrum to show the reaction of *N*-acetyl glycine nitrile (**Ac-Gly-CN**, 200 mM) with L-proline (**Pro**, 200 mM) and *N*-acetyl-L-cysteine (**Ac-Cys-OH**, 60 mM, 0.3 eq.) with MSM (50 mM) as the internal standard after 24 h at pH 7 and 60 °C. ▶ = *N,N'*-diacetyl-L-cystine (formed by aerial oxidation of **Ac-Cys-OH**) and ✖= **Ac-Gly-NH$_2$**.

^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d) *(2-acetamido-1-iminoethyl)proline*, mixture of rotamers [A:B, 60:40], **Ac-Gly^{N}-Pro-OH** (**A**, ●) (partial assignment): δ_H 4.53 (1H, dd, *J* = 8.8, 2.5 Hz, Pro-αH-COOH), 4.30 (1H, AB, *J* = 17.5 Hz, AcNHCH*H*), 4.05 (1H, AB, *J* = 17.5 Hz, AcNHCH*H*), 3.67-3.64 (1H, m, NCH*H*CH_2CH_2), 3.57-3.53 (1H, m, NCH*H*CH_2CH_2), 2.09 (3H, s, H_3C(CO)), (**B**, ●) (partial assignment): δ_H 4.41 (1H, dd, *J* = 8.5, 2.5 Hz, Pro-αH-COOH), 4.37 (1H, AB, *J* = 17.5 Hz, AcNHCH*H*), 4.30 (1H, obs., AcNHCH*H*), 3.79-3.76 (1H, m, NCH*H*CH_2CH_2), 3.72-3.69 (1H, m, NCH*H*CH_2CH_2), 2.11 (3H, s, H_3C(CO)); *L-proline*, **Pro** (■) (partial assignment): δ_H 4.12 (1H, dd, *J* = 8.9, 6.6 Hz, αH-COOH), 3.42 (1H, dt, *J* = 11.6, 7.1 Hz, HNCH*H*CH_2CH_2), 3.33 (1H, dt, *J* = 11.6, 7.1 Hz, HNCH*H*CH_2CH_2); *N-Acetylglycinamide*, **Ac-Gly-NH$_2$** (✖) (partial assignment): δ_H 3.90 (2H, AB obs. CH_2).

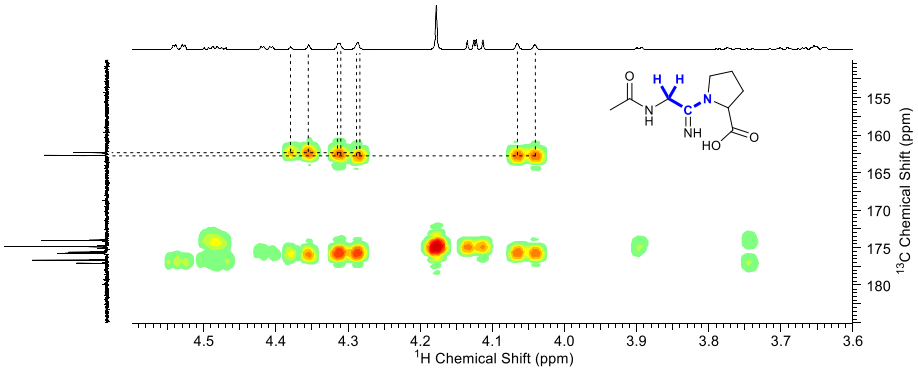
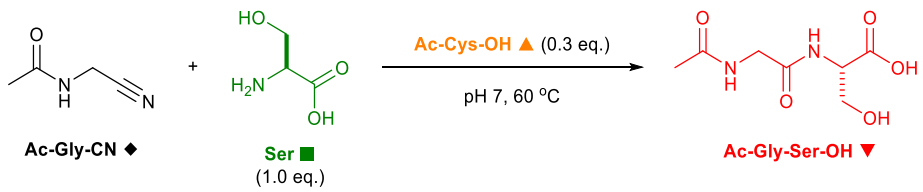


Fig. S115. ^{1}H–^{13}C HMBC (^{1}H: 700 MHz [3.6-4.6 ppm], ^{13}C: 176 MHz [150-180 ppm], H_2O/D_2O 9:1) spectrum showing the diagnostic $^{2}J_{CH}$ coupling of the **Gly**-CH_2 AB systems in both rotamers of **Ac-Gly^{N}-Pro-OH** at 4.38 and 4.35, 4.31 and 4.29 (overlapping AB systems), and 4.07 and 4.04 ppm with two resonances at 162.7 and 162.3 ppm, which is characteristic of amidine bond formation of **Pro**. See Fig. S114 for expanded and labelled ^{1}H NMR spectrum.

Coupling of N-acetylglycine nitrile ***Ac-Gly-CN*** *with L-serine* ***Ser*** *at pH 7 and 60 °C*



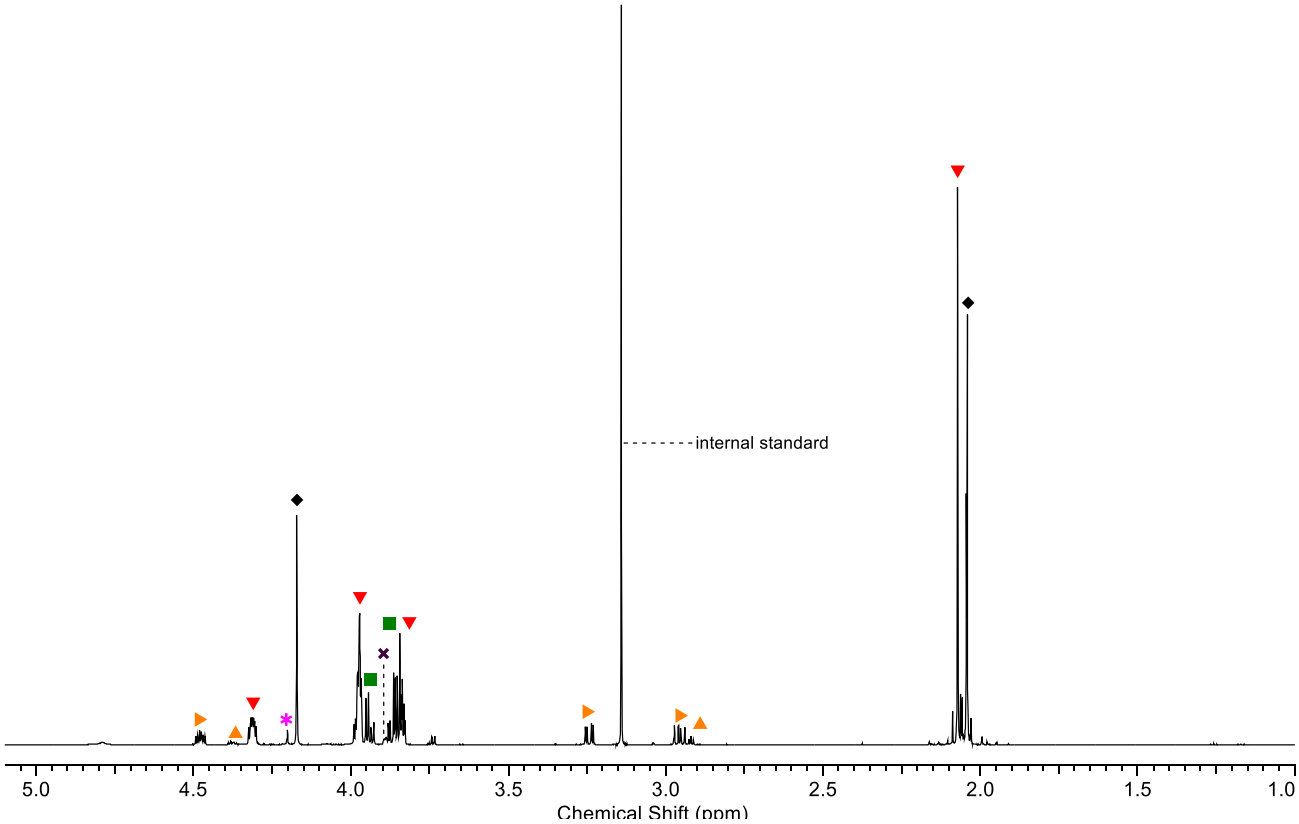


Fig. S116. ^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d, 1.0–5.0 ppm) spectrum to show the reaction of *N*-acetyl glycine nitrile (**Ac-Gly-CN**, 200 mM) with L-serine (**Ser**, 200 mM) and *N*-acetyl-L-cysteine (**Ac-Cys-OH**, 60 mM, 0.3 eq.) with MSM (50 mM) as the internal standard after 24 h at pH 7 and 60 °C. ▶ = *N,N'*-diacetyl-L-cystine (formed by aerial oxidation of **Ac-Cys-OH**) and ✖ = **Ac-Gly-NH$_2$**.

^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d) *N-Acetylglycylserine*, **Ac-Gly-Ser-OH** (▼): δ_H 4.31 (1H, ddd, *J* = 7.6, 5.6, 3.8 Hz, Ser-αH-COOH), 3.98 (2H, obs., AcNHC*H*$_2$), 3.88-3.83 (2H, m, CHC*H*$_2$OH), 2.07 (3H, s, *H*$_3$C(CO)); *L-serine*, **Ser** (■): δ_H 3.99-3.97 (1H, m, CHCH*H*OH), 3.94 (1H, dd, *J* = 12.0, 5.7 Hz, CHCH*H*OH), 3.88-3.83 (1H, m, αH-COOH); *N-Acetylglycinamide*, **Ac-Gly-NH$_2$** (✖) (partial assignment): δ_H 3.89 (2H, obs. br. C*H*$_2$).

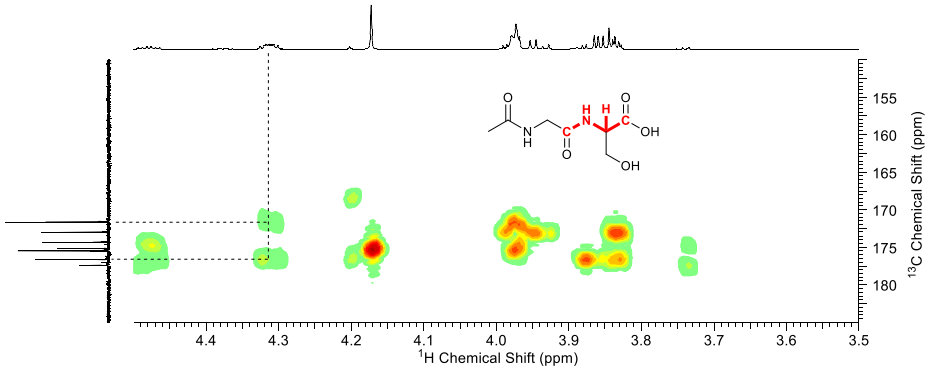


Fig. S117. ^{1}H–^{13}C HMBC (^{1}H: 700 MHz [3.5–4.5 ppm], ^{13}C: 176 MHz [150-185 ppm], H_2O/D_2O 9:1) spectrum showing the diagnostic $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling of the **Ser**-αH-COOH in **Ac-Gly-Ser-OH** at 4.31 ppm with two resonances at 177 and 172 ppm, which is characteristic of amidine bond formation of **Ser**. See Fig. S116 for expanded and labelled ^{1}H NMR spectrum. Stereochemistry on embedded structure is omitted for clarity.

*Coupling of **Ac-Gly-CN** with L-threonine **Thr** at pH 7 and 60 °C*

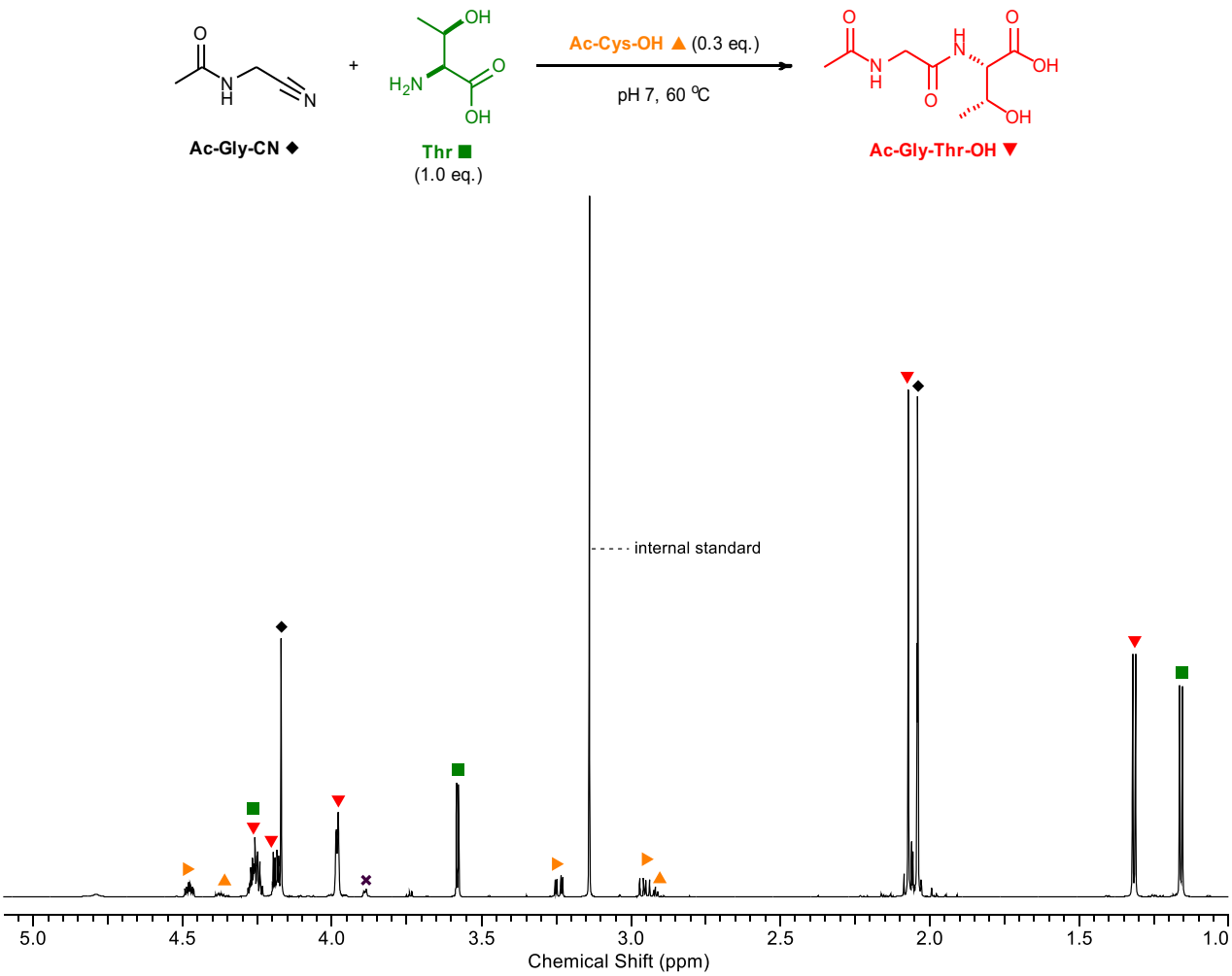


Fig. S118. ^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d, 1.0–5.0 ppm) spectrum to show the reaction of *N*-acetyl glycine nitrile (**Ac-Gly-CN**, 200 mM) with L-threonine (**Thr**, 200 mM) and *N*-acetyl-L-cysteine (**Ac-Cys-OH**, 60 mM, 0.3 eq.) with MSM (50 mM) as the internal standard after 24 h at pH 7 and 60 °C. ▶ = *N,N′*-diacetyl-L-cystine (formed by aerial oxidation of **Ac-Cys-OH**) and ✖ = **Ac-Gly-NH$_2$**.

^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d) *N-Acetylglycylthreonine*, **Ac-Gly-Thr-OH** (▼): δ_H 4.28-4.23 (1H, m, HOC*H*CH$_3$), 4.19 (1H, app. dd, *J* = 8.8, 3.6 Hz, Thr-αH-COOH), 4.00 (1H, AB, *J* = 17.3 Hz, AcNHCH*H*), 3.97 (1H, AB, *J* = 17.3 Hz, AcNHCH*H*), 2.07 (3H, s, *H*$_3$C(CO)), 1.32 (3H, d, *J* = 6.5 Hz, HOCHC*H*$_3$); *L-threonine*, **Thr** (■): δ_H 4.28-4.23 (1H, m, HOC*H*CH$_3$), 3.58 (1H, d, *J* = 4.7 Hz, αH-COOH), 1.16 (3H, d, *J* = 6.5 Hz, HOCHC*H*$_3$); *N-Acetylglycinamide*, **Ac-Gly-NH$_2$** (✖) (partial assignment): δ_H 3.89 (2H, AB obs., C*H*$_2$).

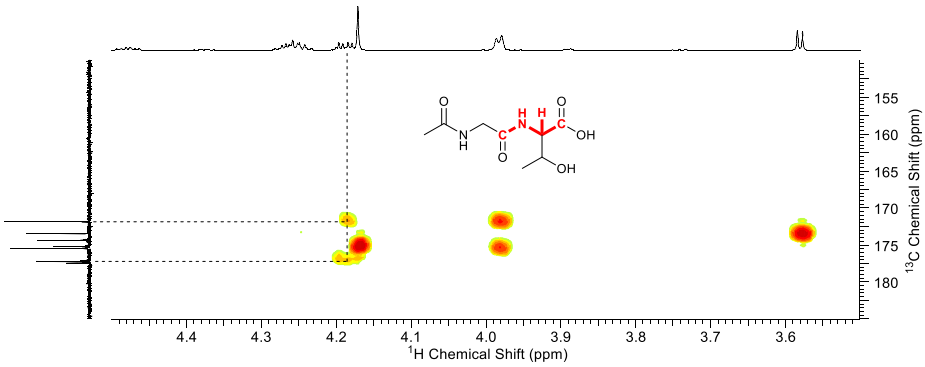


Fig. S119. ^{1}H–^{13}C HMBC (^{1}H: 700 MHz [3.5–4.5 ppm], ^{13}C: 176 MHz [150-185 ppm], H_2O/D_2O 9:1) spectrum showing the diagnostic $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling of the **Thr**-αH-COOH in **Ac-Gly-Thr-OH** at 4.19 ppm with two resonances at 177 and 172 ppm, which is characteristic of peptide bond formation of **Thr**. See Fig. S118 for expanded and labelled ^{1}H NMR spectrum. Stereochemistry on embedded structure is omitted for clarity.

*Coupling of N-acetylglycine nitrile **Ac-Gly-CN** with L-tryptophan **Trp** at pH 7 and 60 °C*

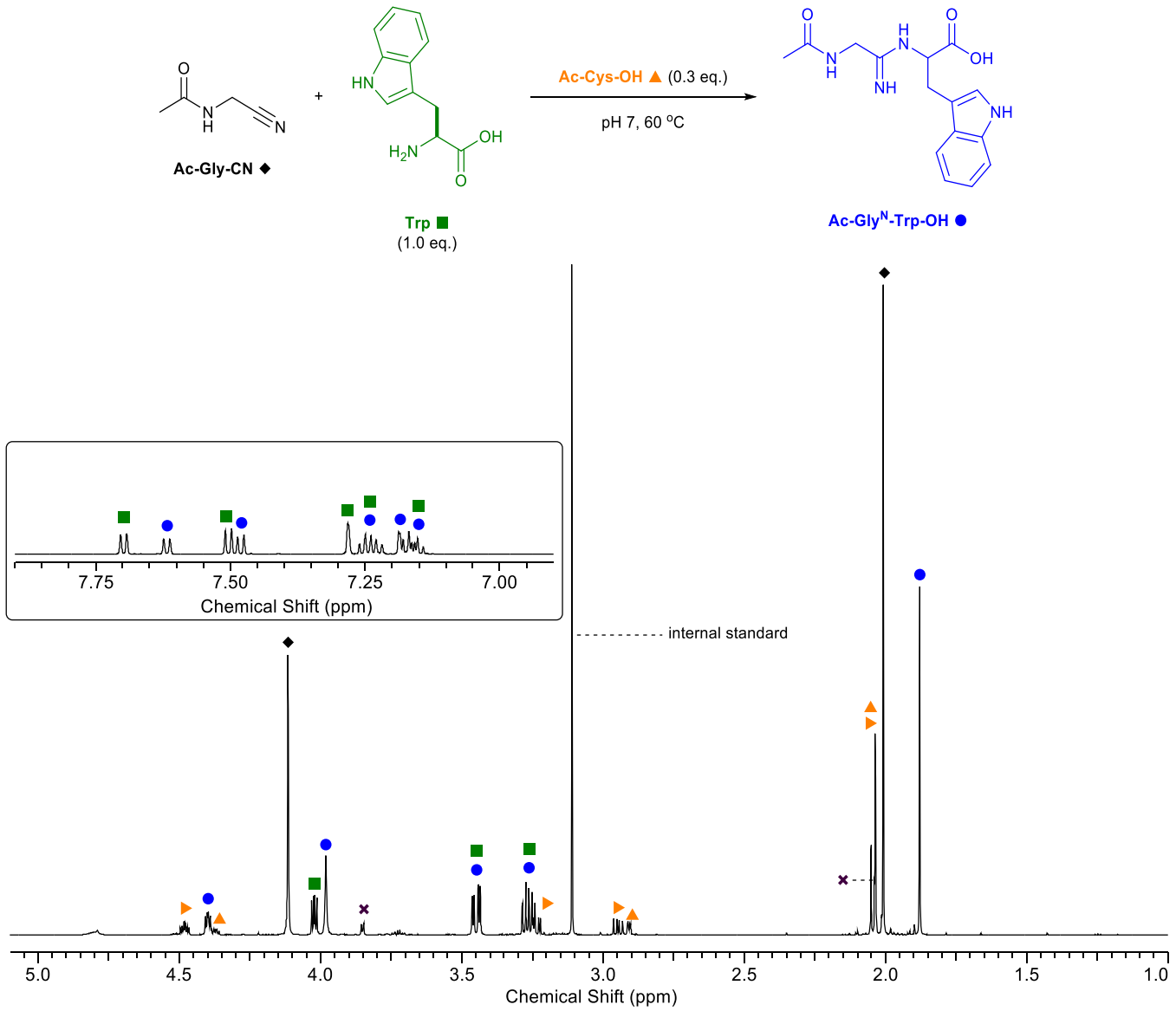


Fig. S120. ^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d, 1.0–5.0 ppm) spectrum to show the reaction of *N*-acetyl glycine nitrile (**Ac-Gly-CN**, 200 mM) with L-tryptophan (**Trp**, 200 mM) and *N*-acetyl-L-cysteine (**Ac-Cys-OH**, 60 mM, 0.3 eq.) with MSM (50 mM) as the internal standard after 24 h at pH 7 and 60 °C. Inset: ^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d, 6.9-7.9 ppm) showing the aromatic CH resonances present in **Trp** and **Ac-Gly^N-Trp-OH**. ▶ = *N,N'*-diacetyl-L-cystine (formed by aerial oxidation of **Ac-Cys-OH**) and ✖= **Ac-Gly-NH$_2$**.

^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d) *(2-acetamido-1-iminoethyl)tryptophan*, **Ac-Gly^N-Trp-OH** (●): δ_H 7.62 (1H, d, *J* = 8.1 Hz, Ar*H*), 7.48 (1H, d, *J* = 8.1 Hz, Ar*H*), 7.26-7.22 (1H, m, Ar*H*), 7.19 (1H, d, *J* = 1.6 Hz, Ar*H*), 7.18-7.14 (1H, m, Ar*H*), 4.40 (1H, ABX, *J* = 7.4, 4.7 Hz, Trp-αH-COOH), 3.98 (2H, s br., AcNHC*H*$_2$), 3.45 (1H, dd, *J* = 15.3, 4.7 Hz, CHCH*H*Ar), 3.29 (1H, m, CHCH*H*Ar), 1.88 (3H, s, *H*$_3$C(CO)); *L-tryptophan*, **Trp** (■): δ_H 7.70 (1H, d, *J* = 8.1 Hz, Ar*H*), 7.50 (1H, d, *J* = 8.3 Hz, Ar*H*), 7.28 (1H, s, Ar*H*), 7.26-7.22 (1H, m, Ar*H*), 7.18-7.14 (1H, m, Ar*H*), 4.02 (1H, ABX, *J* = 8.2, 4.8 Hz, αH-COOH); *N-Acetylglycinamide*, **Ac-Gly-NH$_2$** (✖): δ_H 3.85 (2H, app d., *J* = 4.0 Hz, C*H*$_2$), 2.04 (3H, s, *H*$_3$C(CO)).

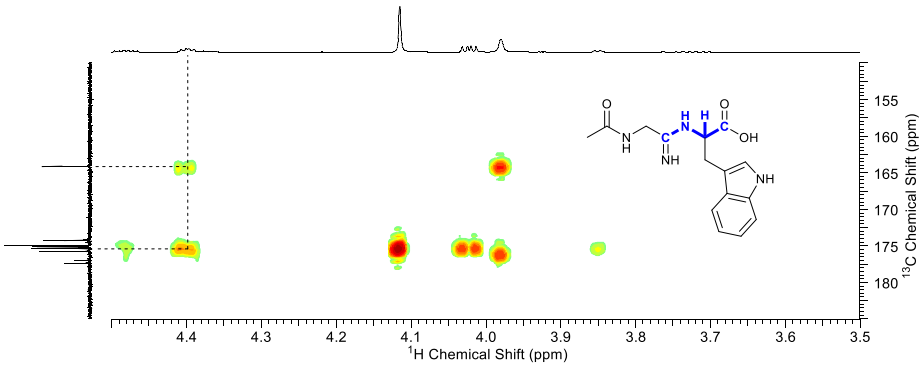


Fig. S121. ^{1}H–^{13}C HMBC (^{1}H: 700 MHz [3.5–4.5 ppm], ^{13}C: 176 MHz [150–185 ppm], H_2O/D_2O 9:1) spectrum showing the diagnostic $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling of the **Trp**-αH-COOH in **Ac-Gly^{N}-Trp-OH** at 4.40 ppm with two resonances at 175 and 164 ppm, which is characteristic of amidine bond formation of **Trp**. See Fig. S120 for expanded and labelled ^{1}H NMR spectrum.

Coupling of N-acetylglycine nitrile ***Ac-Gly-CN*** *with L-tyrosine* ***Tyr*** *at pH 7 and 60 °C*

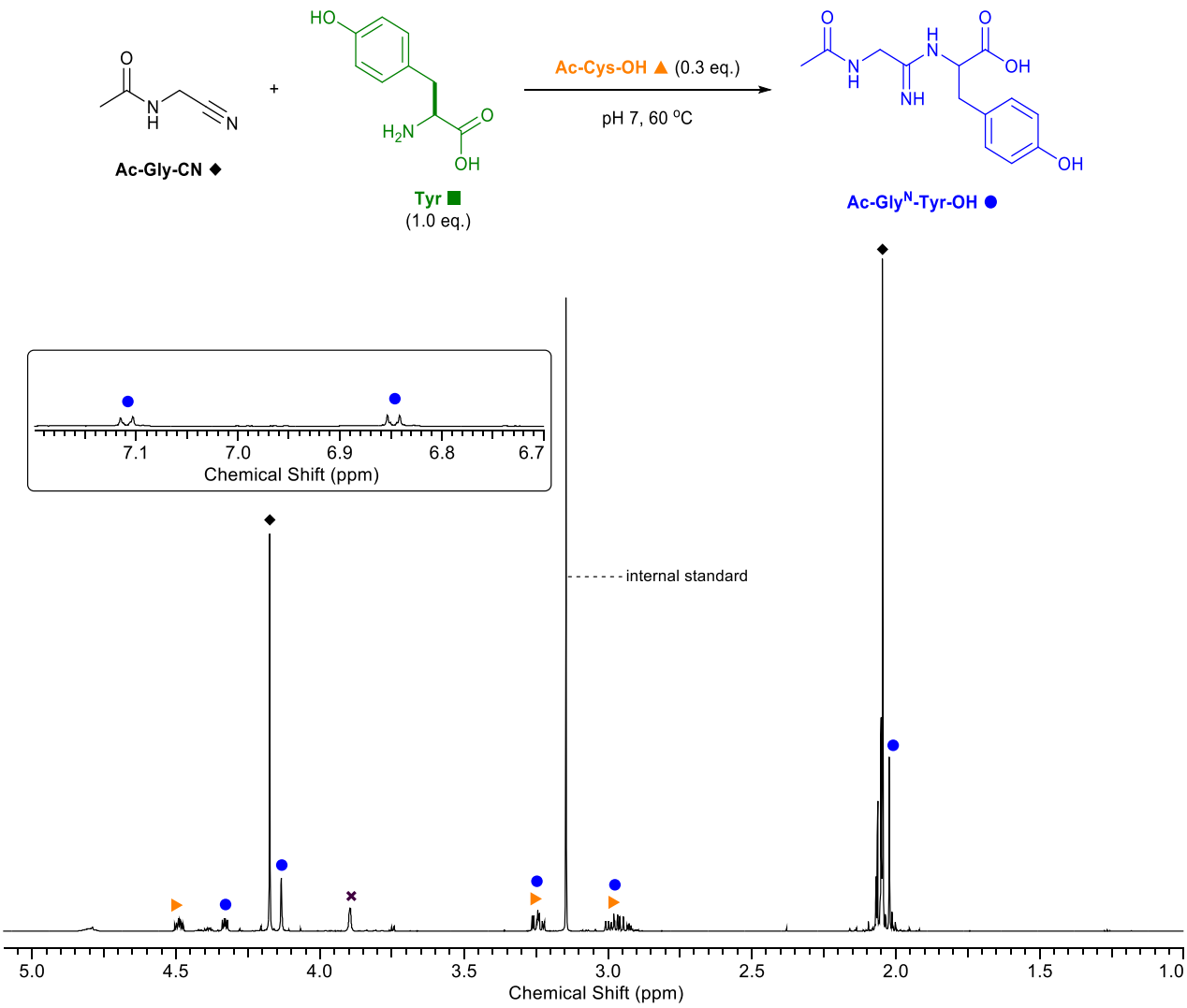


Fig. S122. ^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d, 1.0–5.0 ppm) spectrum to show the reaction of *N*-acetyl glycine nitrile (**Ac-Gly-CN**, 200 mM) with L-tyrosine (**Tyr**, 200 mM) and *N*-acetyl-L-cysteine (**Ac-Cys-OH**, 60 mM, 0.3 eq.) with MSM (50 mM) as the internal standard after 24 h at pH 7 and 60 °C. Residual **Tyr** is not visible by ^{1}H NMR due to its very low solubility in water. See References 50 and 51. Inset: ^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d, 6.7-7.2 ppm) showing the aromatic CH resonances present in **Ac-Gly^{N}-Tyr-OH**. ▶ = *N,N'*-diacetyl-L-cystine (formed by aerial oxidation of **Ac-Cys-OH**) and ✖= **Ac-Gly-NH$_2$**.

^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d) *(2-acetamido-1-iminoethyl)tyrosine*, **Ac-Gly^{N}-Tyr-OH** (●): δ_H 7.11 (2H, d, *J* = 8.5 Hz, Ar*H*), 6.85 (2H, d, *J* = 8.5 Hz, Ar*H*), 4.33 (1H, ABX, *J* = 8.5, 4.6 Hz, Tyr-αH-COOH), 4.13 (2H, s, AcNHC*H*$_2$), 3.25 (1H, dd, *J* = 14.0, 4.6 Hz, CHCH*H*Ar), 2.96 (1H, dd, *J* = 14.0, 8.5 Hz, CHCH*H*Ar), 2.02 (3H, s, *H*$_3$C(CO)); *N-Acetylglycinamide*, **Ac-Gly-NH$_2$** (✖) (partial assignment): δ_H 3.89 (2H, s br., C*H*$_2$).

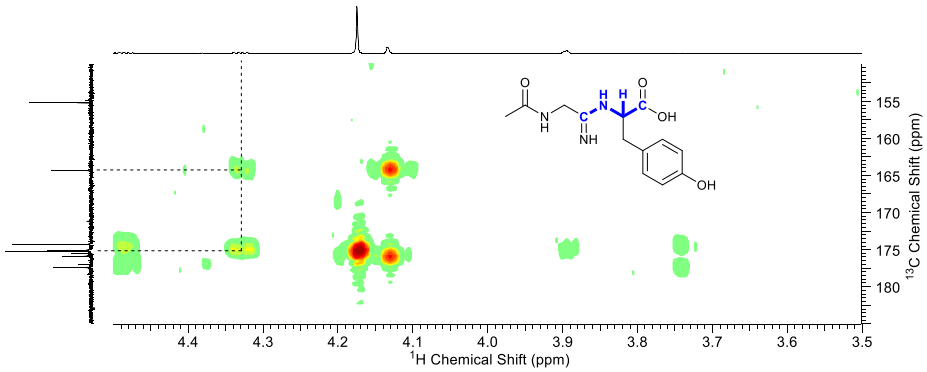


Fig. S123. ^{1}H–^{13}C HMBC (^{1}H: 700 MHz [3.5–4.5 ppm], ^{13}C: 176 MHz [150–185 ppm], H_2O/D_2O 9:1) spectrum showing the diagnostic $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling of the **Tyr**-αH-COOH in **Ac-Gly^{N}-Tyr-OH** at 4.33 ppm with two resonances at 175 and 164 ppm, which is characteristic of amidine bond formation of **Tyr**. See Fig. S122 for expanded and labelled ^{1}H NMR spectrum.

*Coupling of N-acetylglycine nitrile **Ac-Gly-CN** with L-valine **Val** at pH 7 and 60 °C*

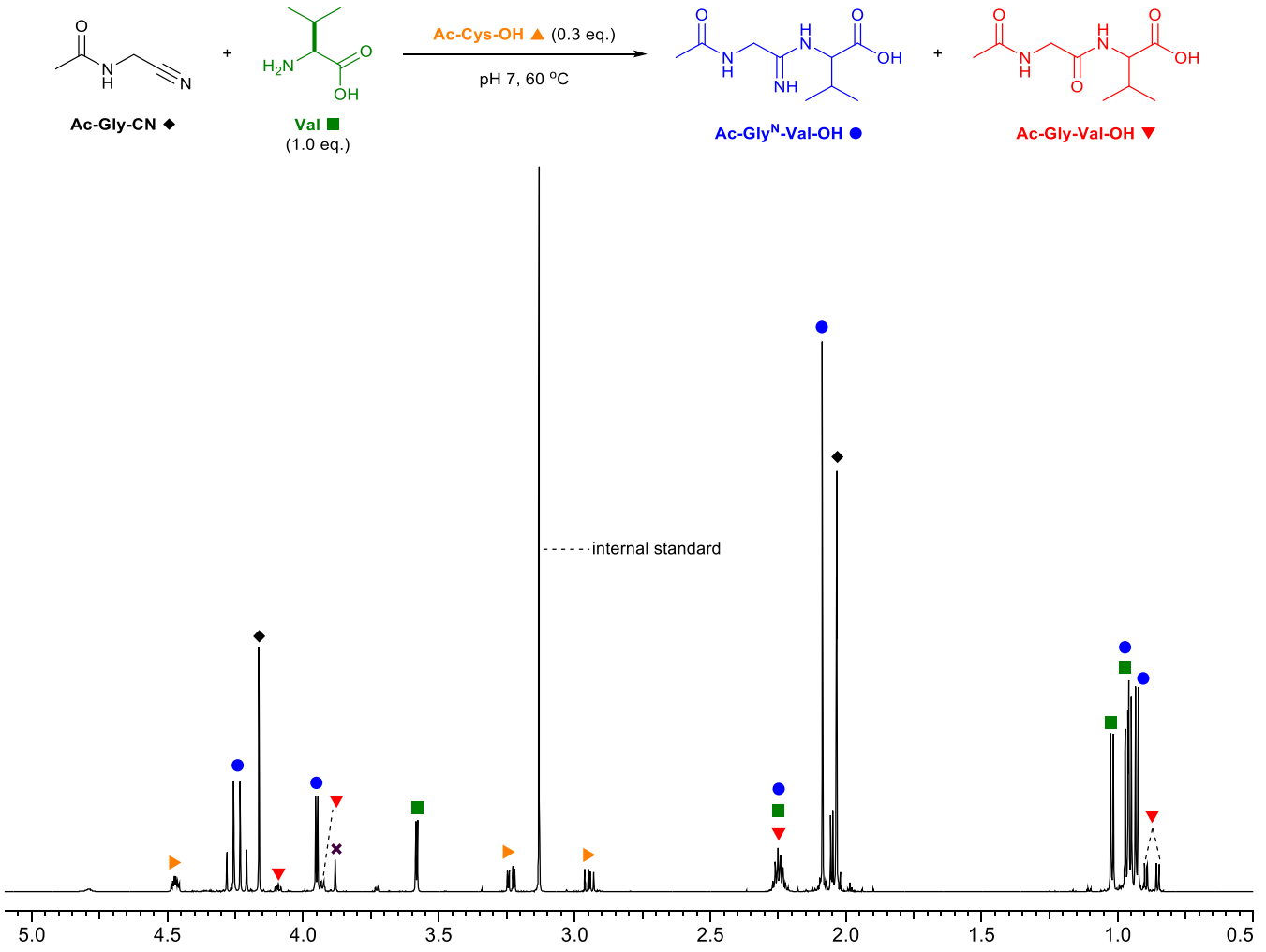


Fig. S124. ^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d, 0.5–5.0 ppm) spectrum to show the reaction of *N*-acetyl glycine nitrile (**Ac-Gly-CN**, 200 mM) with L-valine (**Val**, 200 mM) and *N*-acetyl-L-cysteine (**Ac-Cys-OH**, 60 mM, 0.3 eq.) with MSM (50 mM) as the internal standard after 24 h at pH 7 and 60 °C. ▶ = *N,N'*-diacetyl-L-cystine (formed by aerial oxidation of **Ac-Cys-OH**) and ✖= **Ac-Gly-NH$_2$.**

^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d) *(2-acetamido-1-iminoethyl)valine*, **Ac-Gly^N-Val-OH** (●) (partial assignment): δ_H 4.27 (1H, AB, *J* = 17.1 Hz, AcNHCH*H*), 4.22 (1H, AB, *J* = 17.1 Hz, AcNHCH*H*), 3.95 (1H, d, *J* = 5.6 Hz, Val-αH-COOH), 2.28-2.21 (1H, m, H_3CCHCH_3), 2.09 (3H, s, $H_3C(CO)$), 0.95 (3H, d, *J* = 6.7 Hz, CH_3), 0.93 (3H, d, *J* = 6.7 Hz, CH_3); *N-Acetylglycylvaline*, **Ac-Gly-Val-OH** (▼): δ_H 4.09 (2H, dd, *J* = 8.5, 5.6 Hz, AcNHCH_2), 3.93 (1H, d, *J* = 7.2 Hz, Val-αH-COOH), 2.28-2.21 (1H, m, H_3CCHCH_3), 2.05 (3H, s, $H_3C(CO)$), 0.90 (3H, d, *J* = 7.0 Hz, CH_3), 0.85 (3H, d, *J* = 7.0 Hz, CH_3); *L-valine*, **Val** (■): δ_H 3.58 (1H, d, *J* = 4.3 Hz, αH-COOH), 2.28-2.21 (1H, m, H_3CCHCH_3), 1.02 (3H, d, *J* = 7.0 Hz, CH_3), 0.97 (3H, d, *J* = 7.0 Hz, CH_3); *N-Acetylglycinamide*, **Ac-Gly-NH$_2$** (✖) (partial assignment): δ_H 3.88 (2H, s, CH_2).

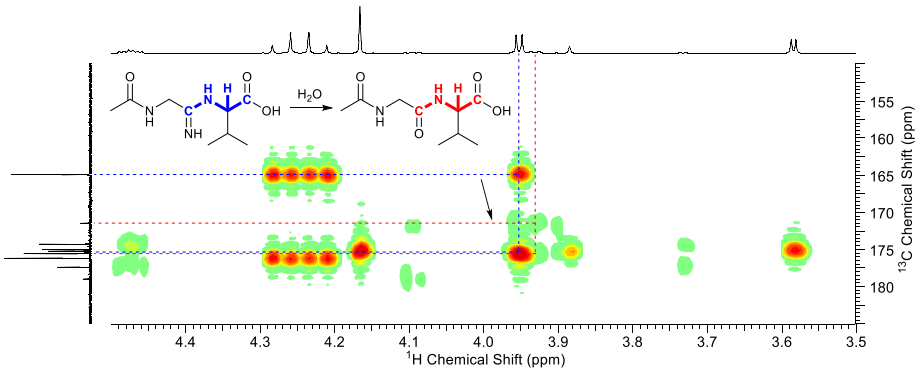


Fig. S125. ^{1}H–^{13}C HMBC (^{1}H: 700 MHz [3.5–4.5 ppm], ^{13}C: 176 MHz [150-185 ppm], H_2O/D_2O 9:1) spectrum showing the diagnostic $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling of the **Val**-αH-COOH in **Ac-Gly^{N}-Val-OH** at 3.95 ppm with two resonances at 176 and 165 ppm, which is characteristic of amidine bond formation of **Val**, and the $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling of **Val**-αH-COOH in **Ac-Gly-Val-OH** at 3.93 ppm with two resonances at 175 and 171 ppm, which is characteristic of peptide bond formation of **Val**. See Fig. S124 for expanded and labelled ^{1}H NMR spectrum.

Coupling of N-acetyl-2-aminoglutaronitrile ***Ac-Glx-CN*** *with glycine* ***Gly*** *at pH 7 and 60 °C*

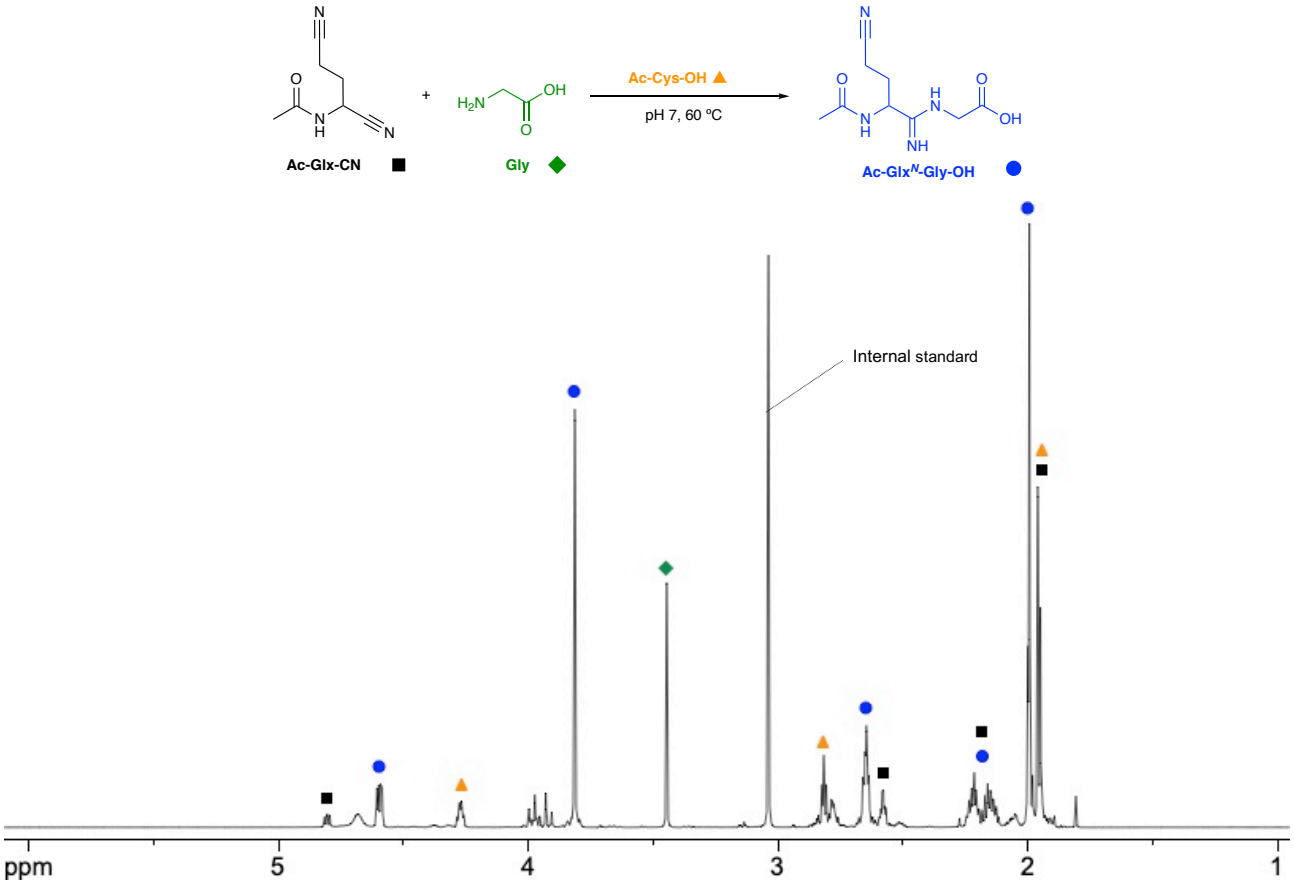


Fig. S126. ^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d, 1.0–5.0 ppm) spectrum to show the reaction of **Ac-Glx-CN** (200 mM) with glycine (**Gly**, 200 mM) and *N*-acetyl-L-cysteine (**Ac-Cys-OH**, 60 mM, 0.3 eq.) with MSM (55 mM) as the internal standard at pH 7 and 60 °C for 4 d.

^{1}H NMR (700 MHz, H_2O/D_2O 9:1, 1–6 ppm): **Ac-Glx^{N}-Gly-OH** (●): δ 4.60 (1H, dd, *J* = 10.0, 4.7 Hz, -C*H*CH$_2$CH$_2$CN), 3.82 (2H, s, Gly-C*H*$_2$), 2.66 (2H, td, *J* = 7.2, 3.4 Hz, -CHCH$_2$C*H*$_2$CN) 2.25–2.13 (2H, m, -CHC*H*$_2$CH$_2$CN), 2.00 (3H, s, *H*$_3$C(CO)-). Glycine **Gly** (◆): δ 3.46 (2H, s, C*H*$_2$).

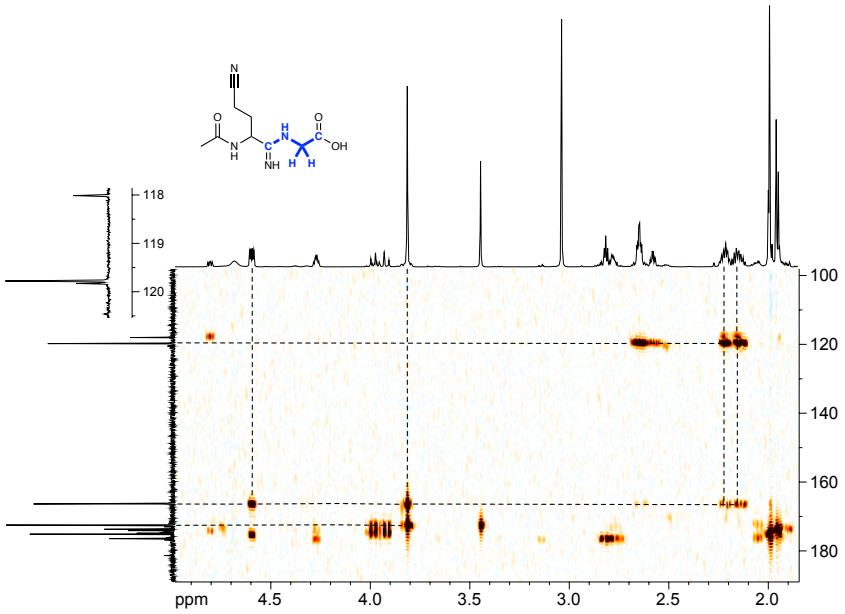


Fig. S127. ^{1}H–^{13}C HMBC (^{1}H: 700 MHz [1.9–4.9 ppm], ^{13}C: 176 MHz [100–190 ppm], H_2O/D_2O 9:1) spectrum showing the diagnostic HMBC coupling between the amidine carbon of **Ac-Glx^{N}-Gly-OH** at 166.4 ppm and its glycyl CH$_2$ moiety at 3.82 ppm, which is characteristic of amidine bond formation of **Gly**. See Fig. S126 for expanded and labelled ^{1}H NMR spectrum.

*Coupling of N-acetyl-DL-alanine nitrile **Ac-Ala-CN** with glycine **Gly** at pH 7 and 60 °C*

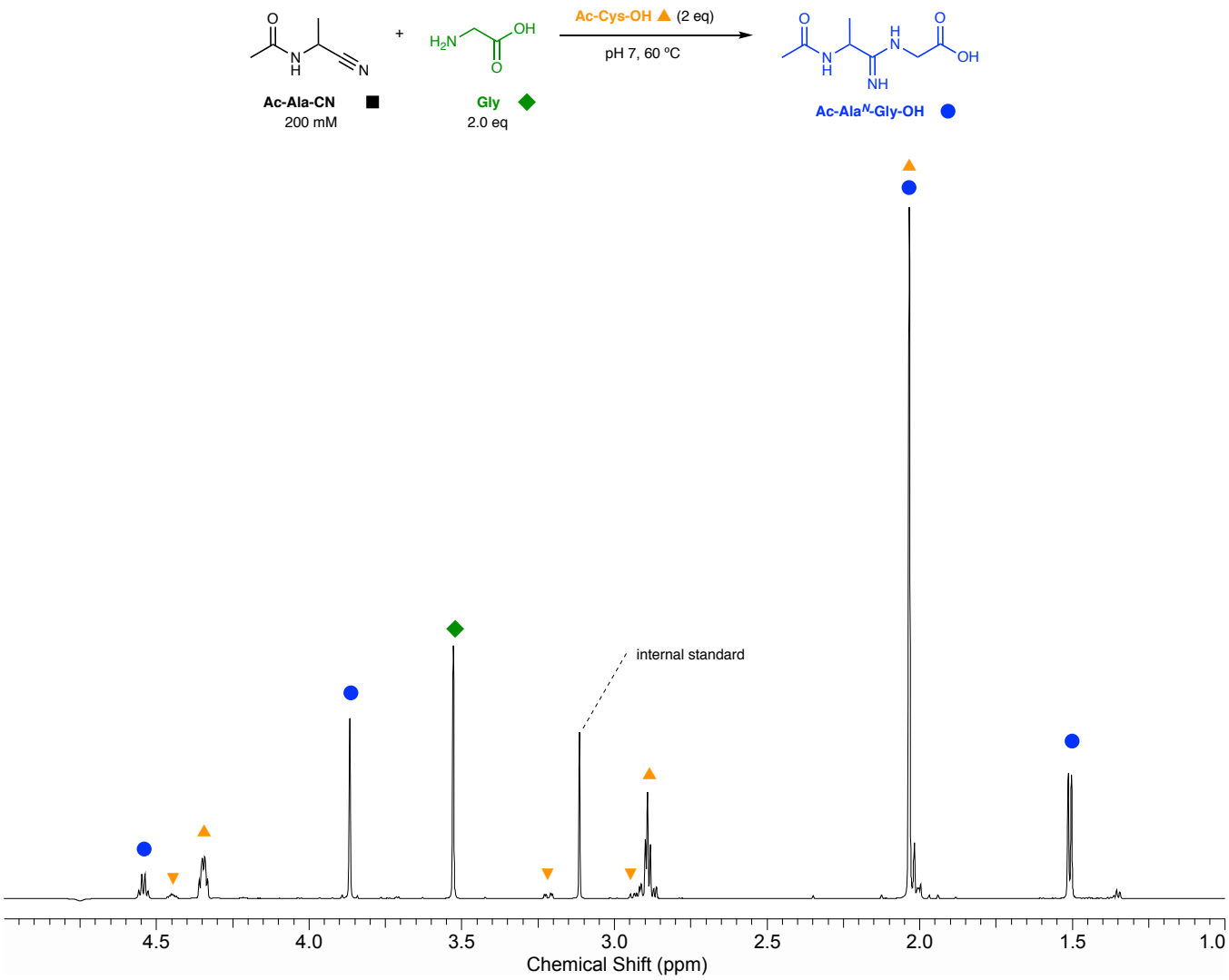


Fig. S128. ^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d, 1.0–6.0 ppm) spectrum showing the coupling of *N*-acetyl-DL-alanine nitrile (**Ac-Ala-CN**, 200 mM) with glycine (**Gly**, 400 mM) catalysed by *N*-acetyl-L-cysteine (**Ac-Cys-OH**, 400 mM) in presence of **MSM** (50 mM; internal NMR standard) after incubation at 60 ºC for 24 h. ▶ = *N,N′*-diacetyl-L-cystine (formed by aerial oxidation of **Ac-Cys-OH**).

^{1}H NMR (700 MHz, H_2O/D_2O 9:1, 1–6 ppm) **Ac-Ala^{N}-Gly-OH** (●): δ 4.48 (1H, q, *J* = 7.2 Hz, C*H*(CH_3)), 3.80 (2H, s, NHCH_2CO_2H), 1.97 (3H, s, H_3C(CO)), 1.45 (3H, d, *J* = 7.2 Hz, CH(CH_3)); glycine **Gly** (◆): δ 3.46 (2H, s, CH_2).

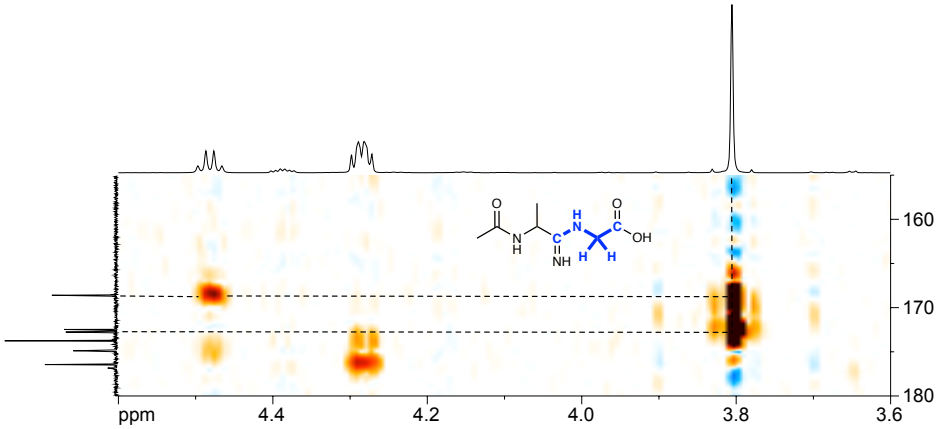


Fig. S129. ^{1}H–^{13}C HMBC (^{1}H: 700 MHz [3.6–4.6 ppm], ^{13}C: 176 MHz [155–180 ppm], H_2O/D_2O 9:1) spectrum showing the diagnostic HMBC coupling between the amidine carbon of **Ac-Ala^{N}-Gly-OH** at 166.4 ppm and its glycyl CH_2 moiety at 3.80 ppm, which is characteristic of amidine bond formation of **Gly**. See Fig. S128 for expanded and labelled ^{1}H NMR spectrum.

*Coupling of N-acetyl-DL-alanine nitrile **Ac-Ala-CN** with L-alanine L-**Ala** at pH 7 and 60 °C*

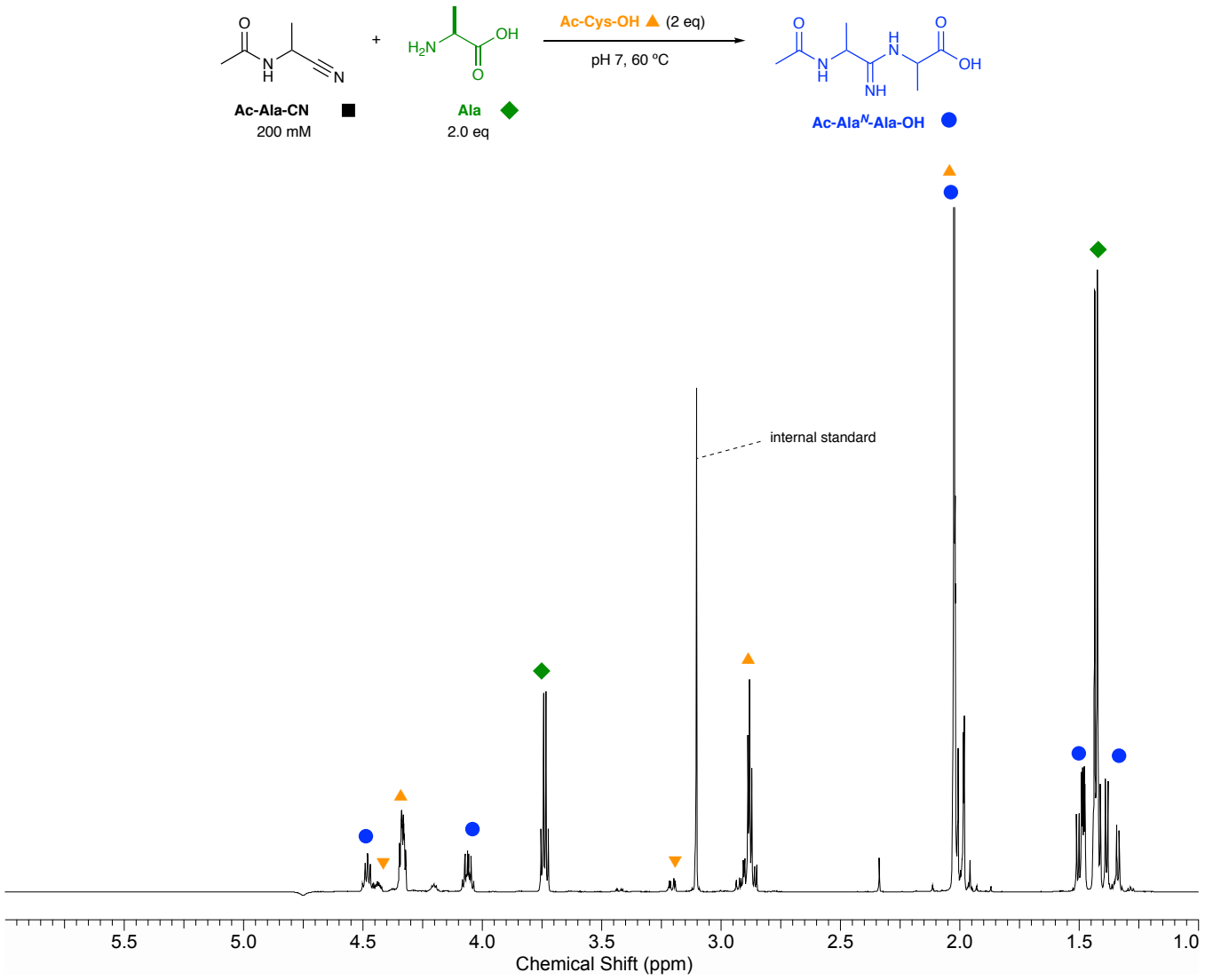


Fig. S130. ^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d, 1.0–6.0 ppm) spectrum showing the coupling of *N*-acetyl-DL-alanine nitrile (**Ac-Ala-CN**, 200 mM) with L-alanine (**Ala**, 400 mM) catalysed by *N*-acetyl-L-cysteine (**Ac-Cys-OH**, 400 mM) in presence of **MSM** (50 mM; internal NMR standard) after incubation at 60 °C for 24 h. ▶ = *N,N'*-diacetyl-L-cystine (formed by aerial oxidation product of **Ac-Cys-OH**).

^{1}H NMR (700 MHz, H_2O/D_2O 9:1, 1–6 ppm) **Ac-Ala^{N}-Ala-OH** (●) (2 diastereoisomers): δ 4.42 (2H, quintet, *J* = 7.7 Hz, C*H*(CH_3)), 4.00 (2H, m, NHC*H*(CH_3)CO_2H), 1.96 (6H, s, H_3C(CO)), 1.42 (m, 6H, NHCH(CH_3)CO_2H), 1.35–1.32 (m, 6H, NHCH(CH_3)CN_2H); **Ala** (◆): δ 3.68 (1H, q, *J* = 7.2 Hz, C*H*(CH_3)), 1.37 (3H, d, *J* = 7.2 Hz, CH(CH_3)).

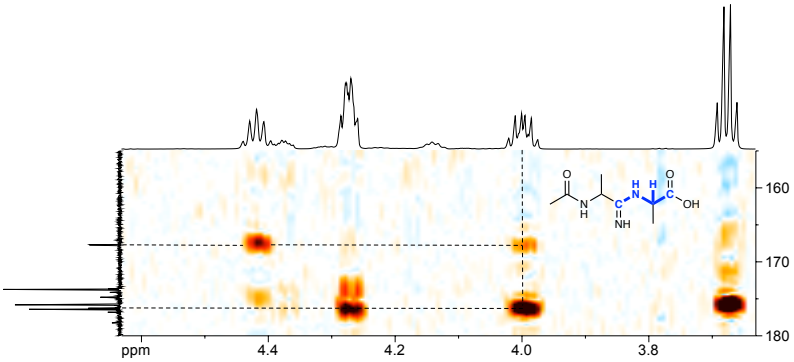


Fig. S131. ^{1}H–^{13}C HMBC (^{1}H: 700 MHz [3.6–4.6 ppm], ^{13}C: 176 MHz [155–180 ppm], H_2O/D_2O 9:1) spectrum showing the diagnostic HMBC coupling between the amidine carbon of **Ac-Ala^{N}-Ala-OH** at 167.7 ppm and its alanyl CH moiety, which is characteristic of amidine bond formation of **Ala**. See Fig. S130 for expanded and labelled ^{1}H NMR spectrum.

Coupling of N-acetyl-DL-serine nitrile ***Ac-Ser-CN*** *with glycine* ***Gly*** *at pH 7 and 60 °C*

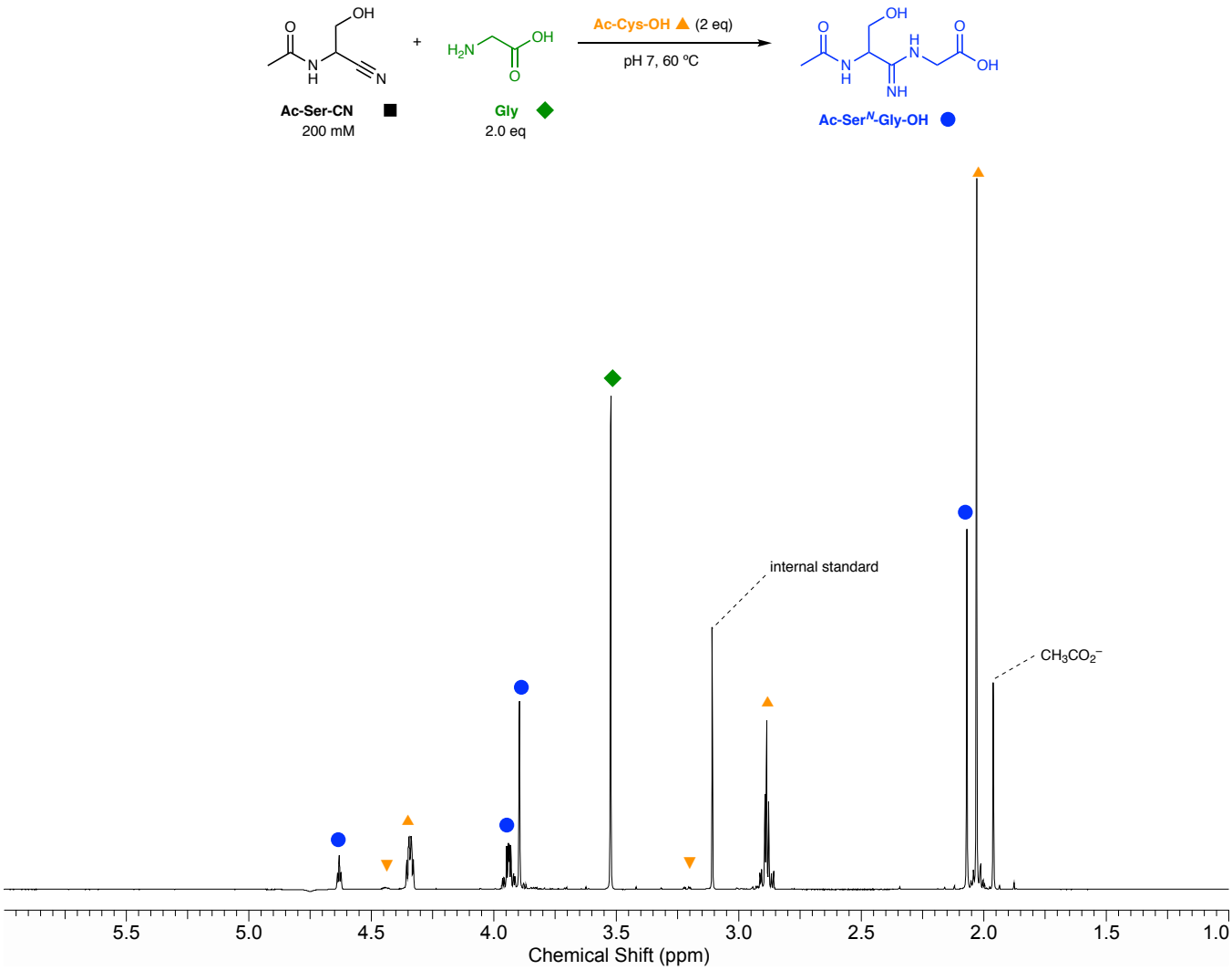


Fig. S132. ^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d, 1.0–6.0 ppm) spectrum showing the coupling of *N*-acetyl-DL-serine nitrile (**Ac-Ser-CN**, 200 mM) with glycine (**Gly**, 400 mM) catalysed by *N*-acetyl-L-cysteine (**Ac-Cys-OH**, 400 mM) in presence of **MSM** (50 mM; internal NMR standard) after incubation at 60 °C for 24 h. ▶ = *N,N′*-diacetyl-L-cystine (formed by aerial oxidation of **Ac-Cys-OH**). Acetate ($CH_3CO_2^-$) was present as a contaminant from **Ac-Ser-CN** synthesis.

^{1}H NMR (700 MHz, H_2O/D_2O 9:1, 1–6 ppm) **Ac-Ser^{N}-Gly-OH** (●): δ 4.67 (1H, t, *J* = 4.7 Hz, C*H*(CH_2OH)), 4.01–3.95 (2H, m, CH(C*H*$_2$OH)), 3.94 (2H, s, NHC*H*$_2$$CO_2$H), 2.11 (3H, s, *H*$_3$C(CO)-); glycine **Gly** (◆): δ (2H, s, $NH_2CH_2CO_2H$).

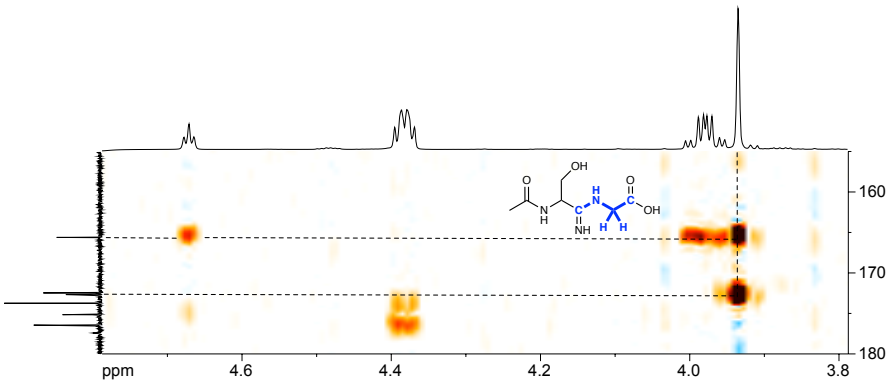


Fig. S133. ^{1}H–^{13}C HMBC (^{1}H: 700 MHz [3.8–4.8 ppm], ^{13}C: 176 MHz [155–180 ppm], H_2O/D_2O 9:1) spectrum showing the diagnostic HMBC coupling between the amidine carbon of **Ac-Ser^{N}-Gly-OH** group at 165.4 ppm and its glycyl CH_2 moiety, which is characteristic of amidine bond formation of **Gly**. See Fig. S132 for expanded and labelled ^{1}H NMR spectrum.

*Coupling of N-acetyl-DL-serine nitrile **Ac-Ser-CN** with L-alanine **Ala** at pH 7 and 60 °C*

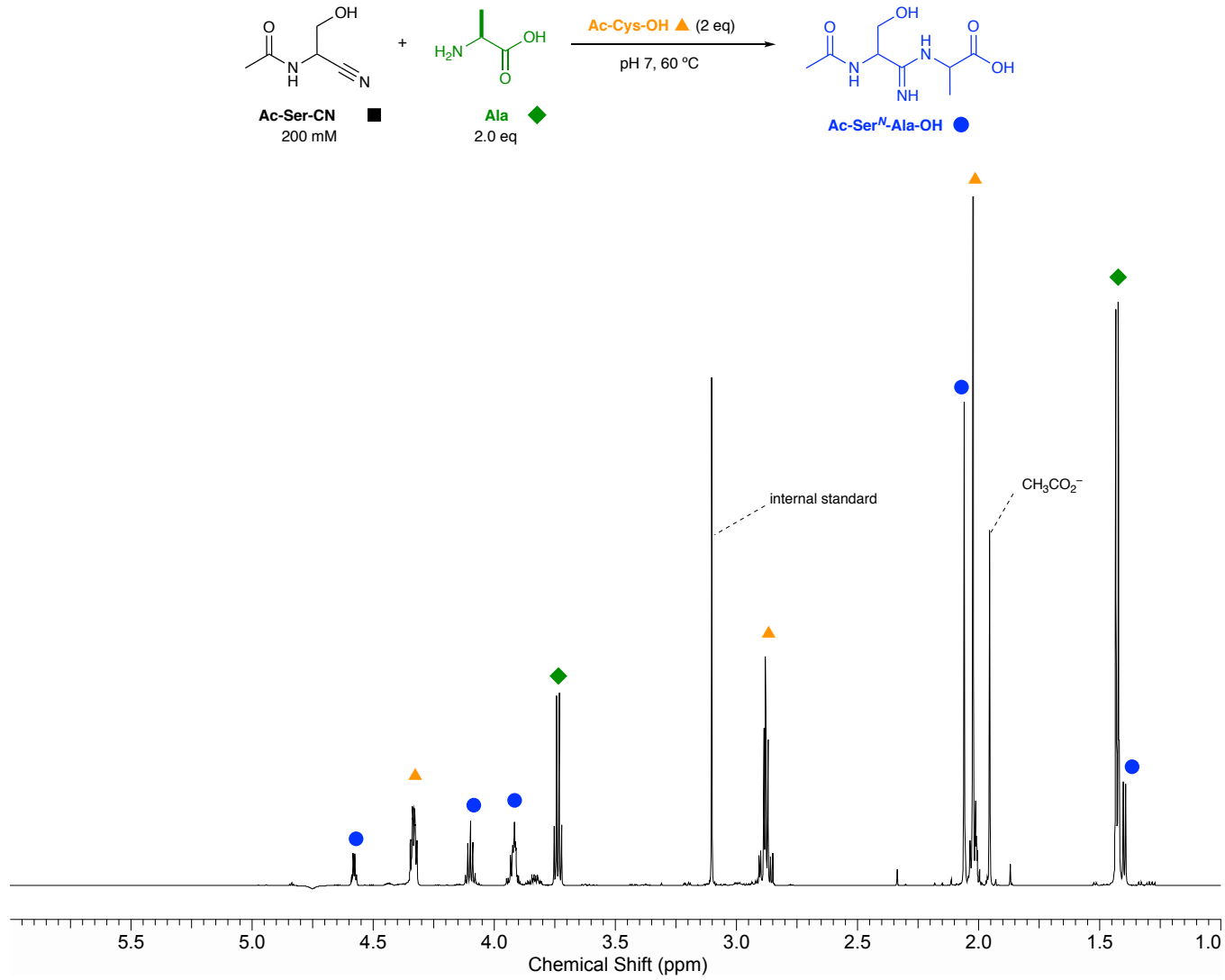


Fig. S134. H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d, 1.0–6.0 ppm) spectrum showing the coupling of *N*-acetyl-DL-serine nitrile (**Ac-Ser-CN**, 200 mM) with L-alanine (**Ala**, 400 mM) catalysed by *N*-acetyl-L-cysteine (**Ac-Cys-OH**, 400 mM) in presence of **MSM** (50 mM; internal NMR standard) after incubation at 60 ºC for 24 h. Acetate ($CH_3CO_2^-$) was present as a contaminant from **Ac-Ser-CN** synthesis.

^{1}H NMR (700 MHz, H_2O/D_2O 9:1, 1–6 ppm) **Ac-Ser^{N}-Ala-OH** (●) (2 diastereoisomers): δ 4.52 (2H, q, *J* = 4.9 Hz, C*H*(CH_2OH)), 4.04 (2H, quintet, *J* = 7.1 Hz, C*H*(CH_3)), 3.89–3.84 (4H, m, CH(C*H*$_2$OH)), 2.00 (6H, s, *H*$_3$C(CO)-), 1.37–1.33 (6H, m, CH(C*H*$_3$)); **Ala** (◆): δ 3.67 (1H, q, *J* = 7.2 Hz, C*H*(CH_3)), 1.36 (3H, d, *J* = 7.2 Hz, CH(C*H*$_3$)).

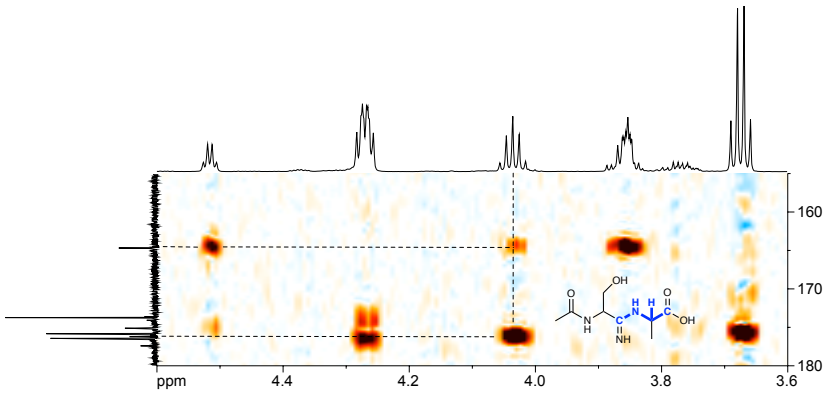


Fig. S135. ^{1}H–^{13}C HMBC (^{1}H: 700 MHz [3.8–4.8 ppm], ^{13}C: 176 MHz [155–180 ppm], H_2O/D_2O 9:1) spectrum showing the diagnostic HMBC coupling between the amidine carbon of **Ac-Ser^{N}-Ala-OH** at 164.7 ppm and its alanyl CH moiety, which is characteristic of amidine bond formation of **Ala**. See Fig. S134 for expanded and labelled ^{1}H NMR spectrum.

Coupling of N-acetyl-DL-valine nitrile ***Ac-Val-CN*** *with glycine* ***Gly*** *at pH 7 and 60 °C*

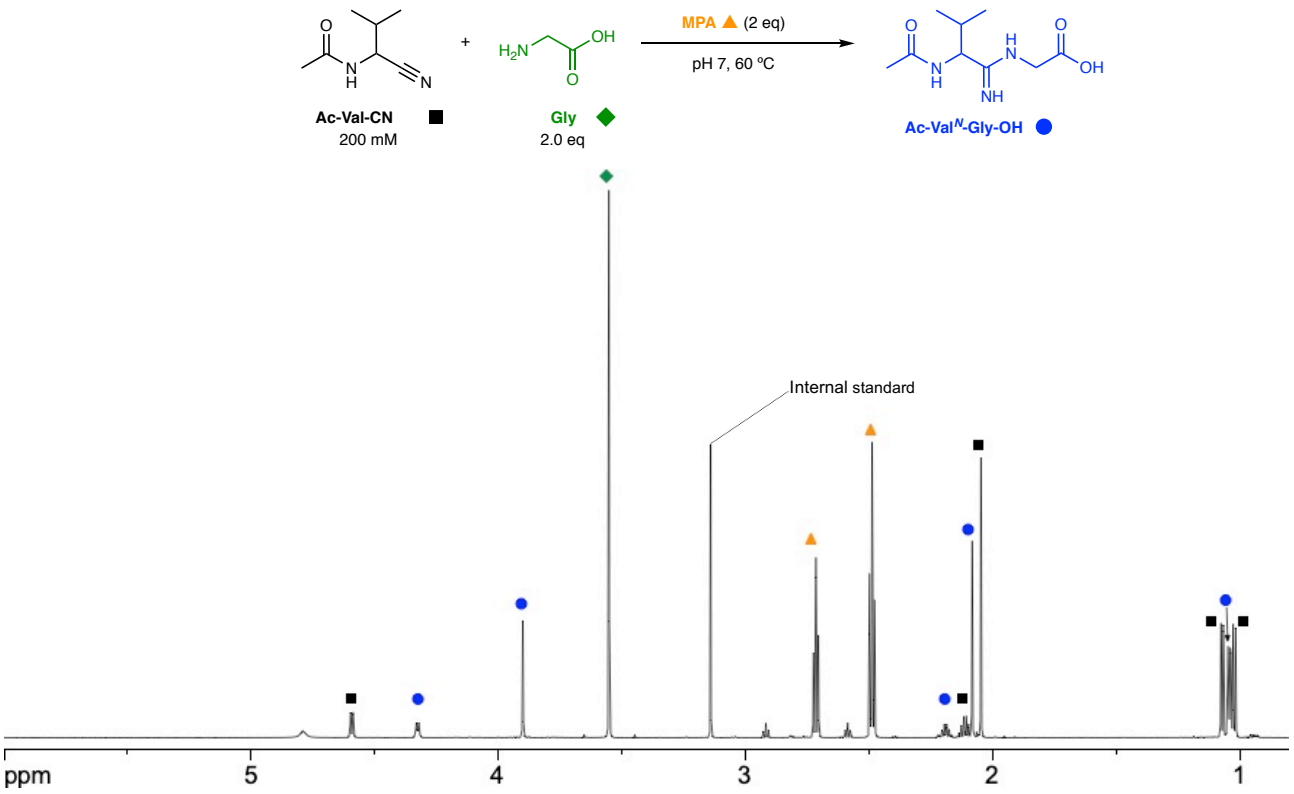


Fig. S136. ^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d, 0.5–6.0 ppm) spectrum showing the coupling of *N*-acetyl-DL-valine nitrile (**Ac-Val-CN**; 200 mM) with glycine (**Gly**; 400 mM) catalysed by 3-mercaptopropionic acid (**MPA**; 400 mM) in presence of **MSM** (50 mM; internal NMR standard) after incubation at 60 °C after 48 h.

^{1}H NMR (700 MHz, H_2O/D_2O 9:1, 1–6 ppm) **Ac-Val^{N}-Gly-OH** (●): δ 4.33 (1H, d, *J* = 6.8 Hz, C*H*CH$(CH_3)_2$), 3.90 (2H, s, NHCH_2CO_2H), 2.19 (1H, m, CHC*H*$(CH_3)_2$), 2.05 (3H, s, H_3C(CO)-), 1.04 (3H, d, *J* = 6.8 Hz, CH(CH_3)(CH_3)), 1.03 (3H, d, *J* = 6.8 Hz, CH(CH_3)(CH_3)); **Gly** (◆): δ 3.55 (s, CH(CH_3)); **Ac-Val-CN** (■): 4.59 (1H, d, *J* = 7.0 Hz, C*H*CH$(CH_3)_2$), 2.13–2.09 (1H, m, CHC*H*$(CH_3)_2$), 2.08 (3H, s, H_3C(CO)-), 1.07 (3H, d, *J* = 6.7 Hz, CHCH(CH_3)(CH_3)), 1.02 (3H, d, *J* = 6.8 Hz, CHCH(CH_3)(CH_3)).

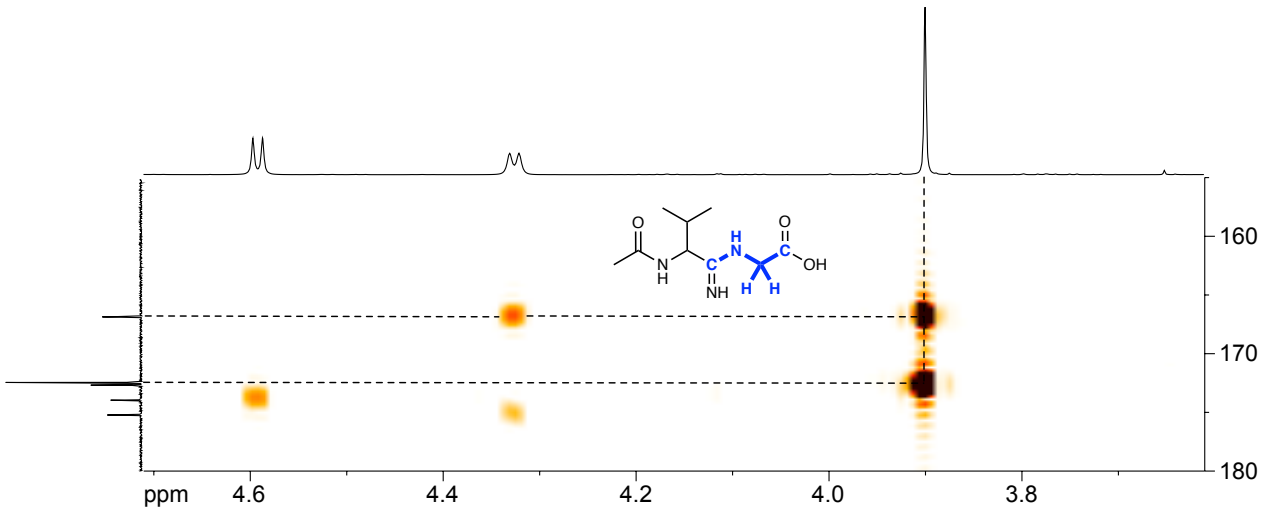


Fig. S137. ^{1}H–^{13}C HMBC (^{1}H: 700 MHz [3.6–4.7 ppm], ^{13}C: 176 MHz [155–180 ppm], H_2O/D_2O 9:1) spectrum showing the diagnostic HMBC coupling between amidine carbon of **Ac-Val^{N}-Gly-OH** at 164.7 ppm and its glycyl CH_2 moiety, which is characteristic of amidine bond formation of **Gly**. See Fig. S136 for expanded and labelled ^{1}H NMR spectrum.

Characterisation of coupling reactions of N-acetyl-L-cysteine-catalysed coupling of N-acetylglycine nitrile and α-amino amides

Coupling of N-acetylglycine nitrile ***Ac-Gly-CN*** *with glycinamide* ***Gly-NH₂*** *at pH 7 and 60 °C*

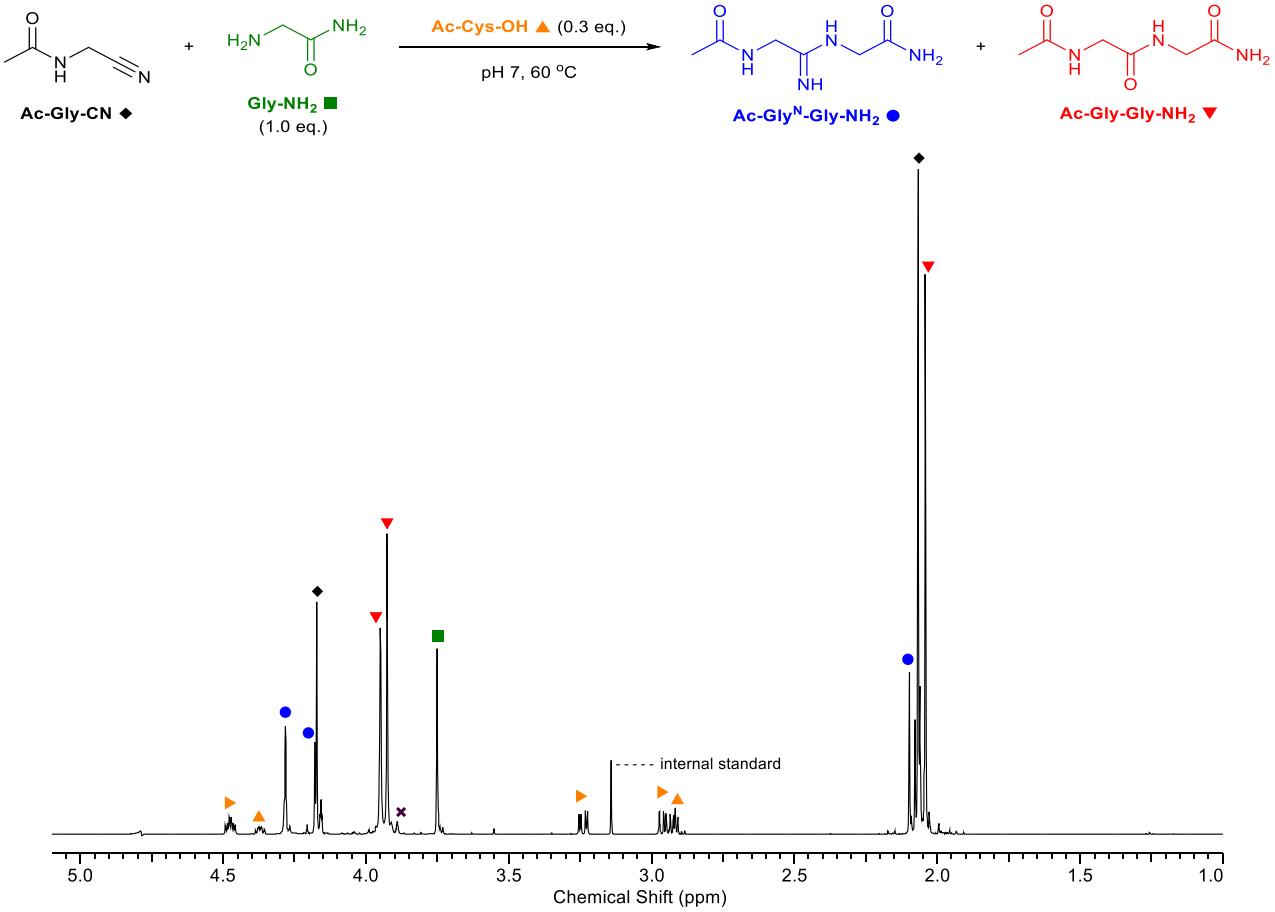


Fig. S138. ^{1}H NMR (600 MHz, H_2O/D_2O 9:1, noesygppr1d, 1.0–5.0 ppm) spectrum to show the reaction of *N*-acetyl glycine nitrile (**Ac-Gly-CN**, 200 mM) with glycinamide (**Gly-NH₂**, 200 mM) and *N*-acetyl-L-cysteine (**Ac-Cys-OH**, 60 mM, 0.3 eq.) with MSM (5 mM) as the internal standard after 24 h at pH 7 and 60 °C. ▶ = *N,N′*-diacetyl-L-cystine (formed by aerial oxidation of **Ac-Cys-OH**) and ✖= **Ac-Gly-NH₂.**

^{1}H NMR (600 MHz, H_2O/D_2O 9:1, noesygppr1d) *(2-acetamido-1-iminoethyl)glycinamide*, **Ac-Gly^{N}-Gly-NH₂** (●): δ_H 4.28 (2H, s, AcNHCH_2), 4.18 (2H, s, CH_2CONH$_2$), 2.10 (3H, s, H_3C(CO)); *N-Acetylglycylglycinamide*, **Ac-Gly-Gly-NH₂** (▼): δ_H 3.95 (2H, s, AcNHCH_2), 3.93 (2H, s, CH_2CONH$_2$), 2.04 (3H, s, H_3C(CO); *Glycinamide*, **Gly-NH₂** (■): δ_H 3.75 (2H, s, CH_2); *N-Acetylglycinamide*, **Ac-Gly-NH₂** (✖) (partial assignment): δ_H 3.89 (2H, s, CH_2).

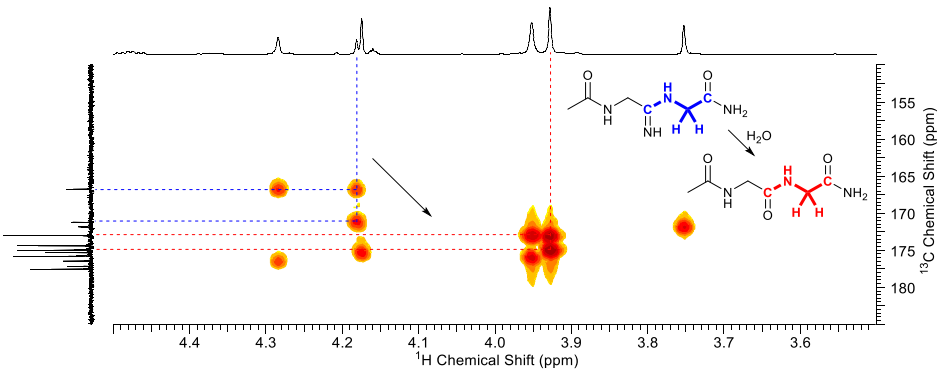


Fig. S139. ^{1}H–^{13}C HMBC (^{1}H: 600 MHz [3.5-4.5 ppm], ^{13}C: 151 MHz [150–185 ppm], H_2O/D_2O 9:1) spectrum showing the diagnostic $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling of the **Gly**-αH-CONH₂ in **Ac-Gly^{N}-Gly-NH₂** at 4.18 ppm with two resonances at 171 and 167 ppm, which is characteristic of amidine bond formation of **Gly-NH₂**, and the diagnostic $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling of the **Gly**-αH-CONH₂ in **Ac-Gly-Gly-NH₂** at 3.93 ppm with two resonances at 175 and 173 ppm, which is characteristic of amide bond formation of **Gly-NH₂**. See Fig. S138 for expanded and labelled ^{1}H NMR spectrum.

Coupling of N-acetylglycine nitrile ***Ac-Gly-CN*** *with D-alaninamide D-****Ala-NH₂*** *at pH 7 and 60 °C*

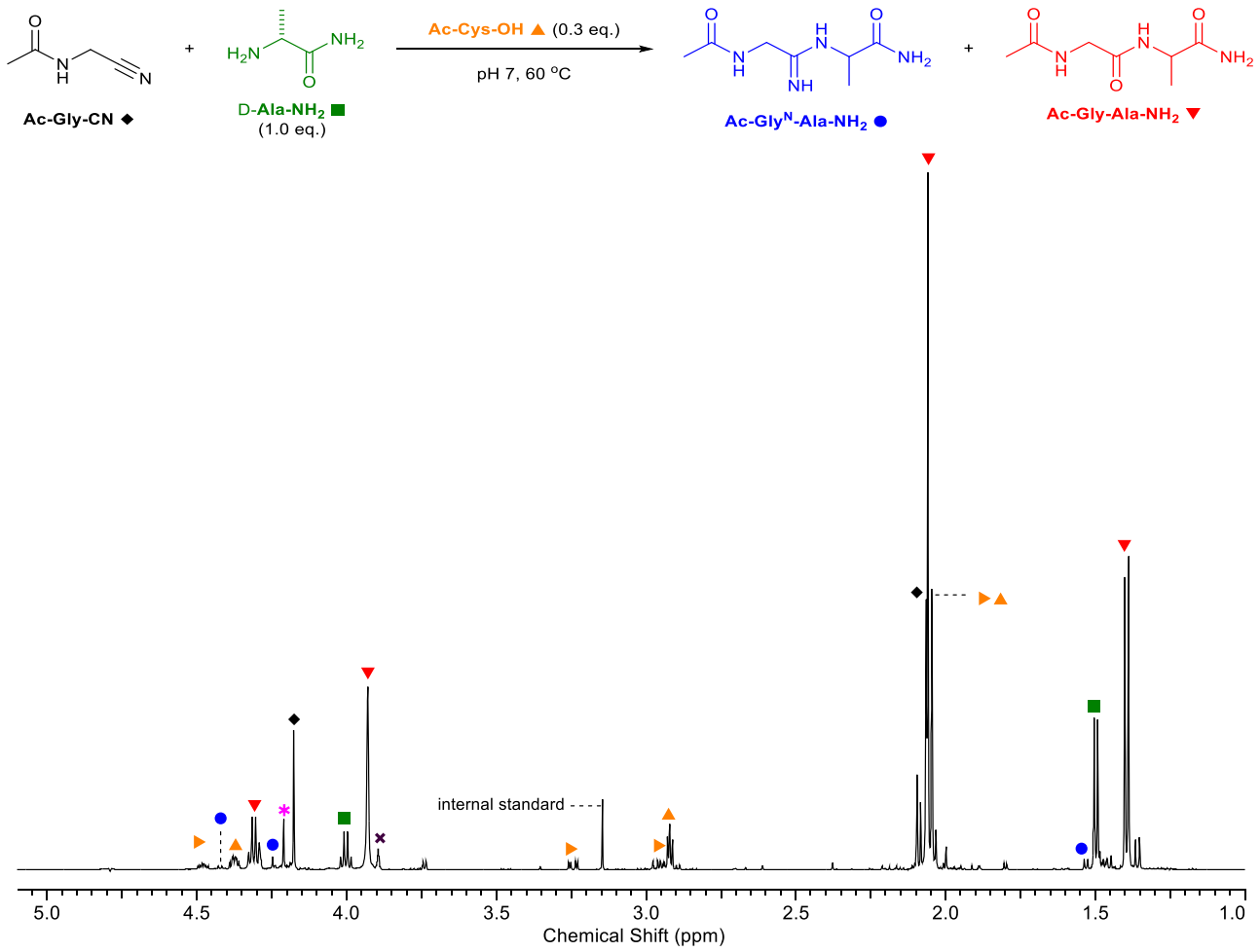


Fig. S140. ^{1}H NMR (600 MHz, H_2O/D_2O 9:1, noesygppr1d, 1.0–5.0 ppm) spectrum to show the reaction of *N*-acetyl glycine nitrile (**Ac-Gly-CN**, 200 mM) with D-alaninamide (D-**Ala-NH₂**, 200 mM) and *N*-acetyl-L-cysteine (**Ac-Cys-OH**, 60 mM, 0.3 eq.) with MSM (5 mM) as the internal standard after 24 h at pH 7 and 60 °C. ▶ = *N,N′*-diacetyl-L-cystine (formed by aerial oxidation of **Ac-Cys-OH**) and ✖= **Ac-Gly-NH₂.**

^{1}H NMR (600 MHz, H_2O/D_2O 9:1, noesygppr1d) *(2-acetamido-1-iminoethyl)alaninamide*, **Ac-Gly^{N}-Ala-NH₂** (●): δ_H 4.42 (1H, q, *J* = 7.0 Hz, C*H*(CH_3), 4.25 (2H, s, AcNHCH_2), 1.53 (3H, d, *J* = 7.0 Hz, CH(CH_3)); *N-Acetylglycylalaninamide*, **Ac-Gly-Ala-NH₂** (▼): δ_H 4.31 (1H, q, *J* = 7.4 Hz, C*H*(CH_3)), 3.93 (2H, br. s., AcNHCH_2), 2.06 (3H, s, H_3C(CO)), 1.40 (3H, d, *J* = 7.4 Hz, CH(CH_3)); D-*Alaninamide*, D-**Ala-NH₂** (■): δ_H 4.00 (1H, q, *J* = 7.2 Hz, C*H*(CH_3)), 1.50 (3H, d, *J* = 7.2 Hz, CH(CH_3)); *N-Acetylglycinamide*, **Ac-Gly-NH₂** (✖) (partial assignment): δ_H 3.89 (2H, s, CH_2); *N-(2-amino-2-iminoethyl)acetamide*, **Ac-Gly^{N}-NH₂** (✱) (partial assignment): δ_H 4.21 (2H, s, CH_2).

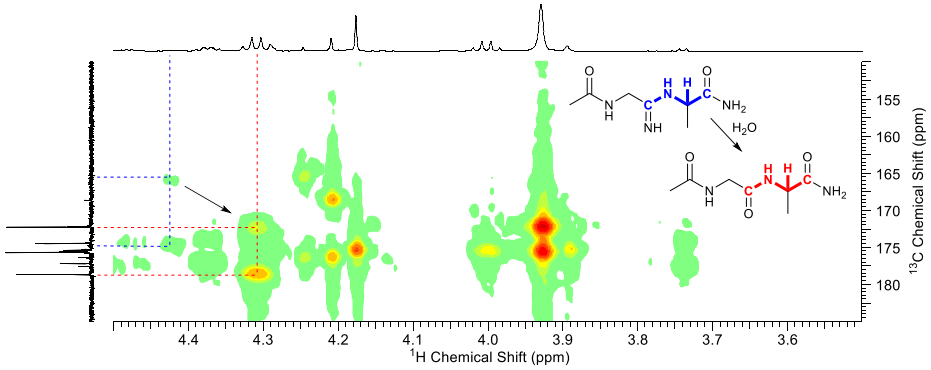


Fig. S141. ^{1}H–^{13}C HMBC (^{1}H: 600 MHz [3.5–4.5 ppm], ^{13}C: 151 MHz [150–185 ppm], H_2O/D_2O 9:1) spectrum showing the diagnostic $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling of the **Ala**-αH-$CONH_2$ in **Ac-Gly^{N}-Ala-NH₂** at 4.42 ppm with two resonances at 175 and 166 ppm, which is characteristic of amidine bond formation of D-**Ala-NH₂** and the diagnostic $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling of the **Ala**-αH-$CONH_2$ in **Ac-Gly-Ala-NH₂** at 4.31 ppm with two resonances at 179 and 172 ppm, which is characteristic of amide bond formation of D-**Ala-NH₂**. See Fig. S140 for expanded and labelled ^{1}H NMR spectrum.

Coupling of N-acetylglycine nitrile ***Ac-Gly-CN*** *with L-argininamide* ***Arg-NH₂*** *at pH 7 and 60 °C*

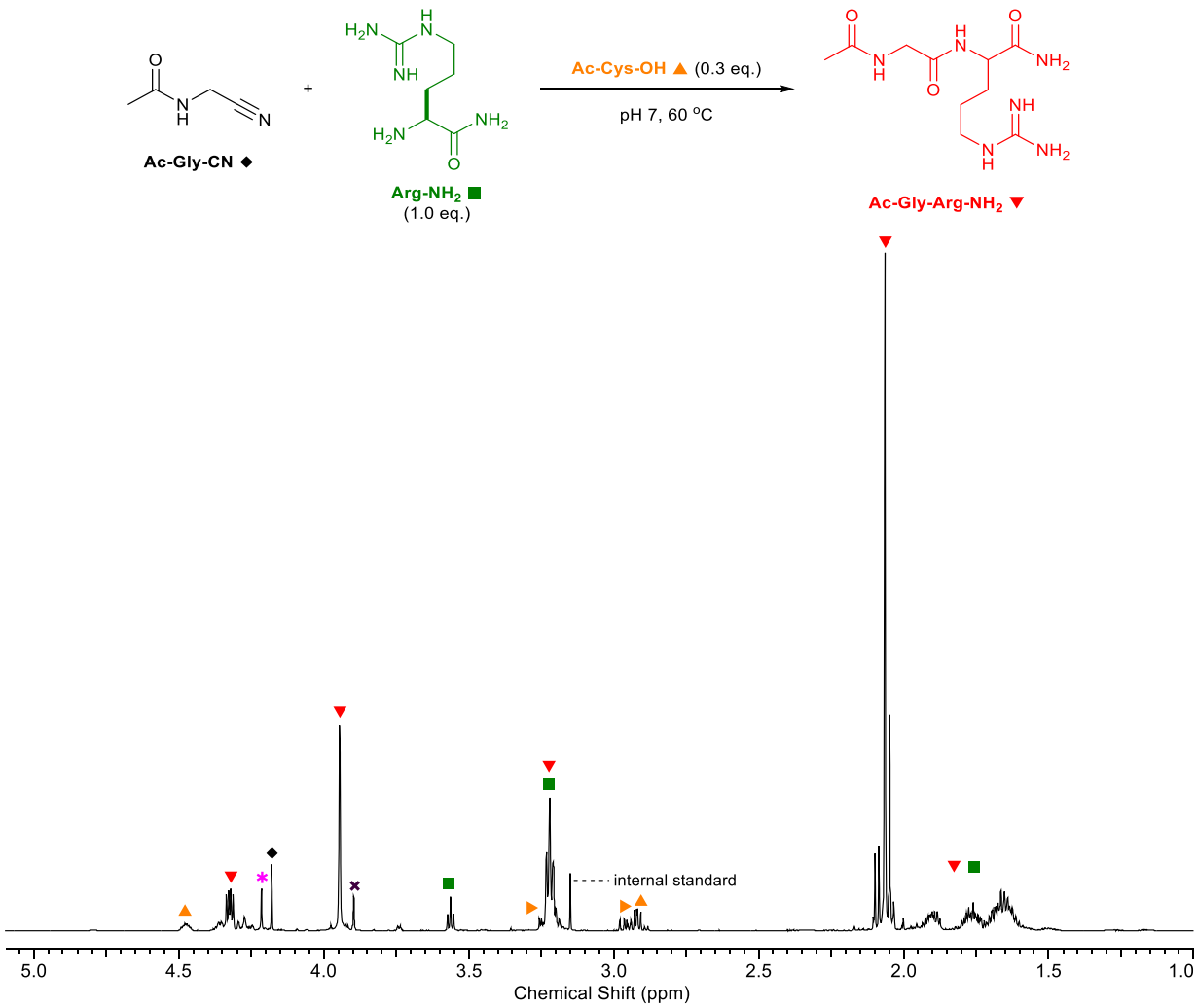


Fig. S142. ^{1}H NMR (600 MHz, H_2O/D_2O 9:1, noesygppr1d, 1.0–5.0 ppm) spectrum to show the reaction of *N*-acetyl glycine nitrile (**Ac-Gly-CN**, 200 mM) with L-argininamide (**Arg-NH₂**, 200 mM) and *N*-acetyl-L-cysteine (**Ac-Cys-OH**, 60 mM, 0.3 eq.) with MSM (5 mM) as the internal standard after 24 h at pH 7 and 60 °C. ▶ = *N,N′*-diacetyl-L-cystine (formed by aerial oxidation of **Ac-Cys-OH**) and ✖= **Ac-Gly-NH₂.**

^{1}H NMR (600 MHz, H_2O/D_2O 9:1, noesygppr1d) *N-Acetylglycylargininamide*, **Ac-Gly-Arg-NH₂** (▼) (partial assignment): δ_H 4.32 (1H, dd, *J* = 9.2, 5.0 Hz, Arg-αH-$CONH_2$), 3.95 (2H, s br., AcNHCH_2), 3.22 (2H, br. t., *J* = 7.0 Hz, CH_2(guanidyl)), 2.06 (3H, s, H_3C(CO)); *L-argininamide*, **Arg-NH₂** (■) (partial assignment): δ_H 3.56 (1H, t, *J* = 6.4 Hz, αH-$CONH_2$), 3.22 (2H, br. t., *J* = 7.0 Hz, CH_2(guanidyl)); *N-Acetylglycinamide*, **Ac-Gly-NH₂** (✖) (partial assignment): δ_H 3.90 (2H, s, CH_2); *N-(2-amino-2-iminoethyl)acetamide*, **Ac-Gly^{N}-NH₂** (✱) (partial assignment): δ_H 4.21 (2H, s, CH_2).

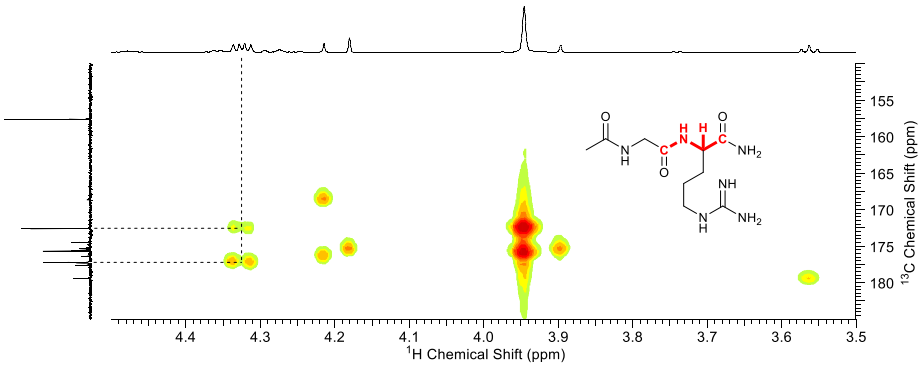


Fig. S143. ^{1}H–^{13}C HMBC (^{1}H: 600 MHz [3.5–4.5 ppm], ^{13}C: 151 MHz [150–185 ppm], H_2O/D_2O 9:1) spectrum showing the diagnostic $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling of the **Arg**-αH-$CONH_2$ in **Ac-Gly-Arg-NH₂** at 4.32 ppm with two resonances at 177 and 173 ppm, which is characteristic of amide bond formation of **Arg-NH₂**. See Fig. S142 for expanded and labelled ^{1}H NMR spectrum.

Coupling of N-acetylglycine nitrile ***Ac-Gly-CN*** *with L-asparaginamide* ***Asn-NH₂*** *at pH 7 and 60 °C*

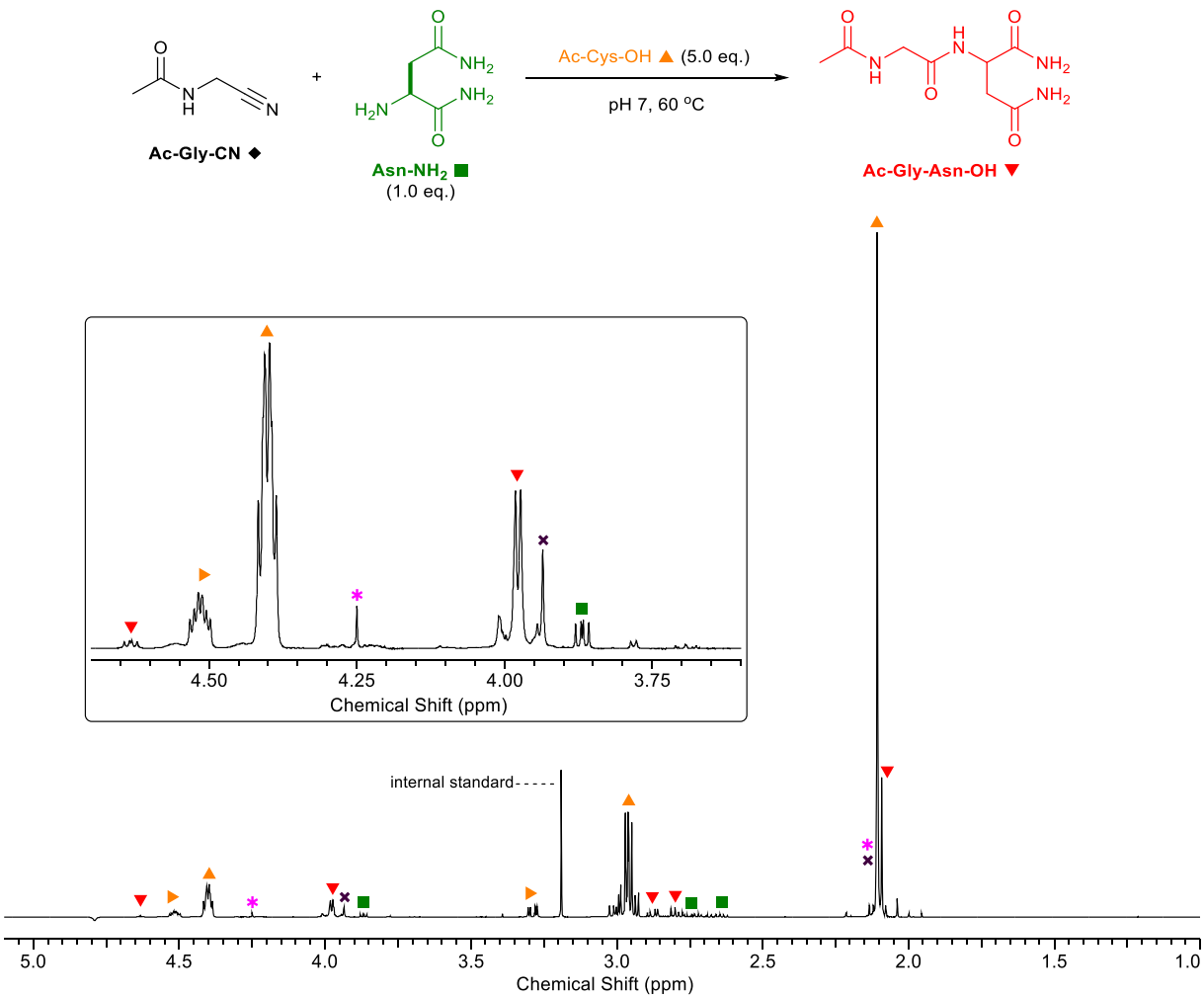


Fig. S144. ^{1}H NMR (600 MHz, H_2O/D_2O 9:1, noesygppr1d, 1.0–5.0 ppm) spectrum to show the reaction of *N*-acetyl glycine nitrile (**Ac-Gly-CN**, 200 mM) with L-asparaginamide (**Asn-NH₂**, 200 mM) and *N*-acetyl-L-cysteine (**Ac-Cys-OH**, 300 mM) with MSM (50 mM) as the internal standard after 24 h at pH 7 and 60 °C. Inset: ^{1}H NMR (600 MHz, H_2O/D_2O 9:1, noesygppr1d, 3.7-4.7 ppm) showing the region with the (C2)–H. The **Asn**-αH (C2)–H resonance of **Ac-Gly-Asn-NH₂** at 4.63 ppm has become suppressed along with the residual HOD peak, but is confirmed by ^{1}H–^{13}C HMBC analysis. See Fig. S145. ▶ = *N,N'*-diacetyl-L-cystine (formed by aerial oxidation of **Ac-Cys-OH**), ✖ = **Ac-Gly-NH₂** and ✱ = **Ac-Gly^{N}-NH₂.**

^{1}H NMR (600 MHz, H_2O/D_2O 9:1, noesygppr1d) *N-Acetylglycylasparaginamide*, **Ac-Gly-Asn-NH₂** (▼): δ_H 4.63 (1H, ABX, *J* = 7.6, 5.3 Hz, Asn-αH-$CONH_2$), 4.00 (1H, AB, *J* = 16.9 Hz, AcNHCH*H*), 3.96 (1H, AB, *J* = 16.9 Hz, AcNHCH*H*), 2.88 (1H, dd, *J* = 15.3, 5.3 Hz, CH(CH*H*$CONH_2$)$CONH_2$), 2.79 (1H, dd, *J* = 15.3 Hz, 7.6 Hz, CH(CH*H*$CONH_2$)$CONH_2$), 2.09 (3H, s, H_3C(CO)); *L-asparaginamide*, **Asn-NH₂** (■): δ_H 3.87 (1H, ABX, *J* = 7.8, 5.6 Hz, αH-$CONH_2$), 2.75 (1H, dd, *J* = 15.3, 5.6 Hz, CH(CH*H*$CONH_2$)$CONH_2$), 2.64 (1H, dd, *J* = 15.3, 7.8 Hz, CH(CH*H*$CONH_2$)$CONH_2$); *N-Acetylglycinamide*, **Ac-Gly-NH₂** (✖) (partial assignment): δ_H 3.93 (2H, s, CH_2); *N-(2-amino-2-iminoethyl)acetamide*, **Ac-Gly^{N}-NH₂** (✱) (partial assignment): δ_H 4.25 (2H, s, CH_2).

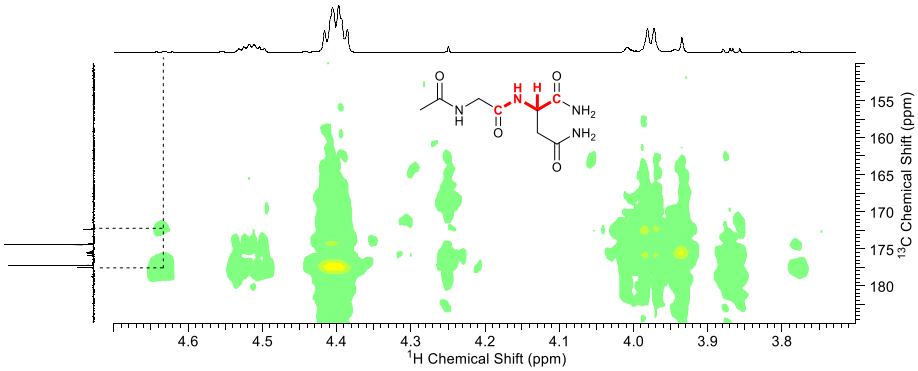


Fig. S145. ^{1}H–^{13}C HMBC (^{1}H: 600 MHz [3.7–4.7 ppm], ^{13}C: 176 MHz [150–185 ppm], H_2O/D_2O 9:1) spectrum showing the diagnostic $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling of the **Asn**-αH-$CONH_2$ in **Ac-Gly-Asn-NH_2** at 4.63 ppm with two resonances at 177 and 172 ppm, which is characteristic of amide bond formation of **Asn-NH_2**. See Fig. S144 for expanded and labelled ^{1}H NMR spectrum.

Coupling of N-acetylglycine nitrile ***Ac-Gly-CN*** *with L-aspartamide* ***Asp-NH₂*** *at pH 7 and 60 °C*

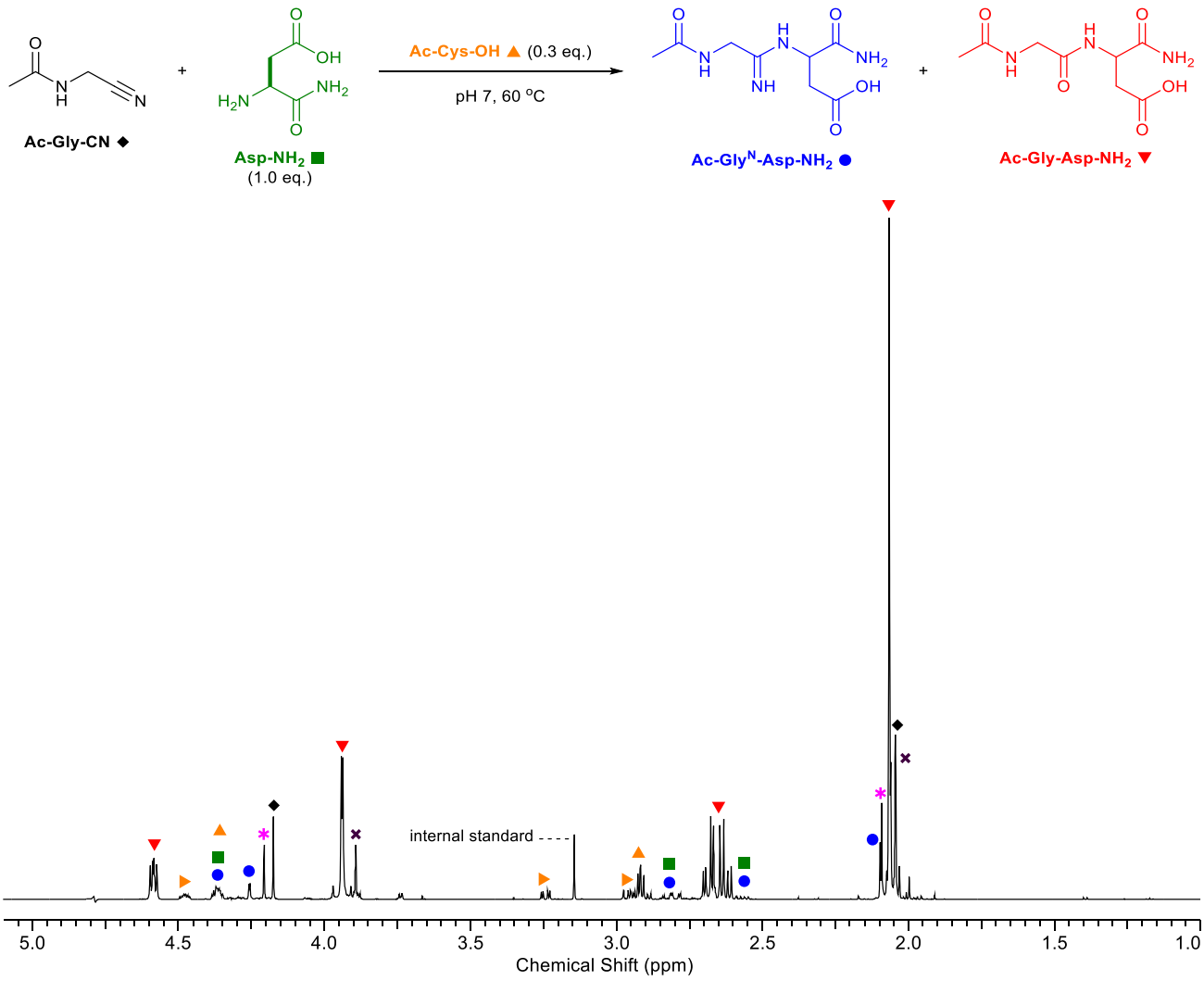


Fig. S146. ^{1}H NMR (600 MHz, H_2O/D_2O 9:1, noesygppr1d, 1.0-5.0 ppm) spectrum to show the reaction of *N*-acetyl glycine nitrile (**Ac-Gly-CN**, 200 mM) with L-aspartamide (**Asp-NH₂**, 200 mM) and *N*-acetyl-L-cysteine (**Ac-Cys-OH**, 60 mM, 0.3 eq.) with MSM (5 mM) as the internal standard after 24 h at pH 7 and 60 °C. ▶ = *N,N'*-diacetyl-L-cystine (formed by aerial oxidation of **Ac-Cys-OH**), ✖= **Ac-Gly-NH₂** and ✱= **Ac-Gly^{N}-NH₂.**

^{1}H NMR (600 MHz, H_2O/D_2O 9:1, noesygppr1d) *(2-acetamido-1-iminoethyl)aspartamide*, **Ac-Gly^{N}-Asp-NH₂** (●) (partial assignment): δ_H 4.38-4.35 (1H, m, Asp-αH-$CONH_2$), 4.27 (1H, AB, *J* = 17.3 Hz, AcNHCH*H*), 4.24 (1H, AB, *J* = 17.3 Hz, AcNHCH*H*), 2.10 (3H, s, $H_3C(CO)$); *N-Acetylglycylaspartamide*, **Ac-Gly-Asp-NH₂** (▼) (partial assignment): δ_H 4.59 (1H, ABX, *J* = 7.7, 5.2 Hz, Asp-αH-$CONH_2$), 3.96 (1H, AB, *J* = 17.1 Hz, AcNHCH*H*), 3.92 (1H, AB, *J* = 17.1 Hz, AcNHCH*H*), 2.69 (1H, dd, *J* = 16.0, 5.2 Hz, CH(CH*H*COOH)$CONH_2$), 2.63 (1H, dd, *J* = 16.0, 7.7 Hz, CH(CH*H*COOH)$CONH_2$), 2.07 (3H, s, $H_3C(CO)$); *L-aspartamide*, **Asp-NH₂** (■) (partial assignment): δ_H 4.38-4.35 (1H, m, αH-$CONH_2$); *N-Acetylglycinamide*, **Ac-Gly-NH₂** (✖): δ_H 3.89 (2H, s, CH_2), 2.06 (3H, s, $H_3C(CO)$); *N-(2-amino-2-iminoethyl)acetamide*, **Ac-Gly^{N}-NH₂** (✱): δ_H 4.21 (2H, s, CH_2), 2.09 (3H, s, $H_3C(CO)$).

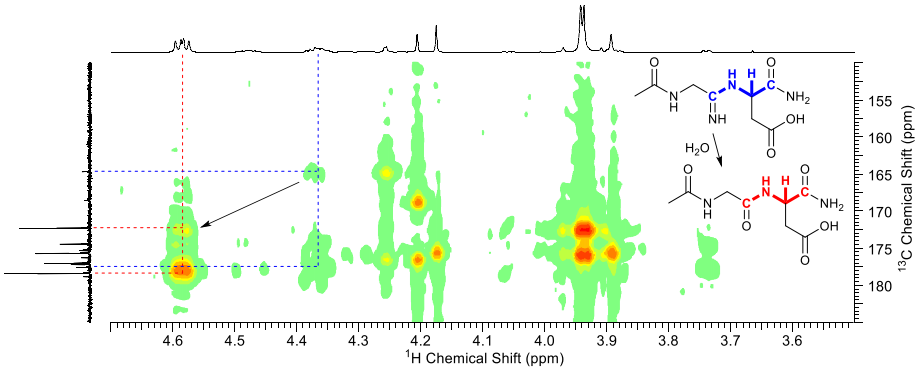
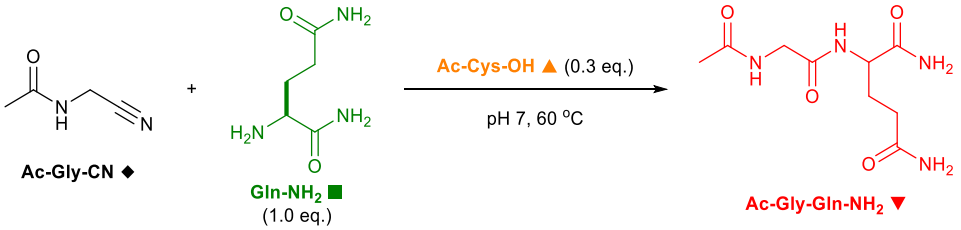


Fig. S147. ^{1}H–^{13}C HMBC (^{1}H: 600 MHz [3.5–4.7 ppm], ^{13}C: 151 MHz [150–185 ppm], H_2O/D_2O 9:1) spectrum showing the diagnostic $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling of the **Asp**-αH-$CONH_2$ in **Ac-Gly^{N}-Asp-NH$_2$** at 4.38-4.35 ppm with two resonances at 178 and 165 ppm, which is characteristic of amidine bond formation of **Asp-NH$_2$**, and the diagnostic $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling of the **Asp**-αH-$CONH_2$ in **Ac-Gly-Asp-NH$_2$** at 4.59 ppm with two resonances at 178 and 172 ppm, which is characteristic of amide bond formation of **Asp-NH$_2$**. See Fig. S146 for expanded and labelled ^{1}H NMR spectrum.

Coupling of N-acetylglycine nitrile ***Ac-Gly-CN*** *with L-glutaminamide* ***Gln-NH₂*** *at pH 7 and 60 °C*



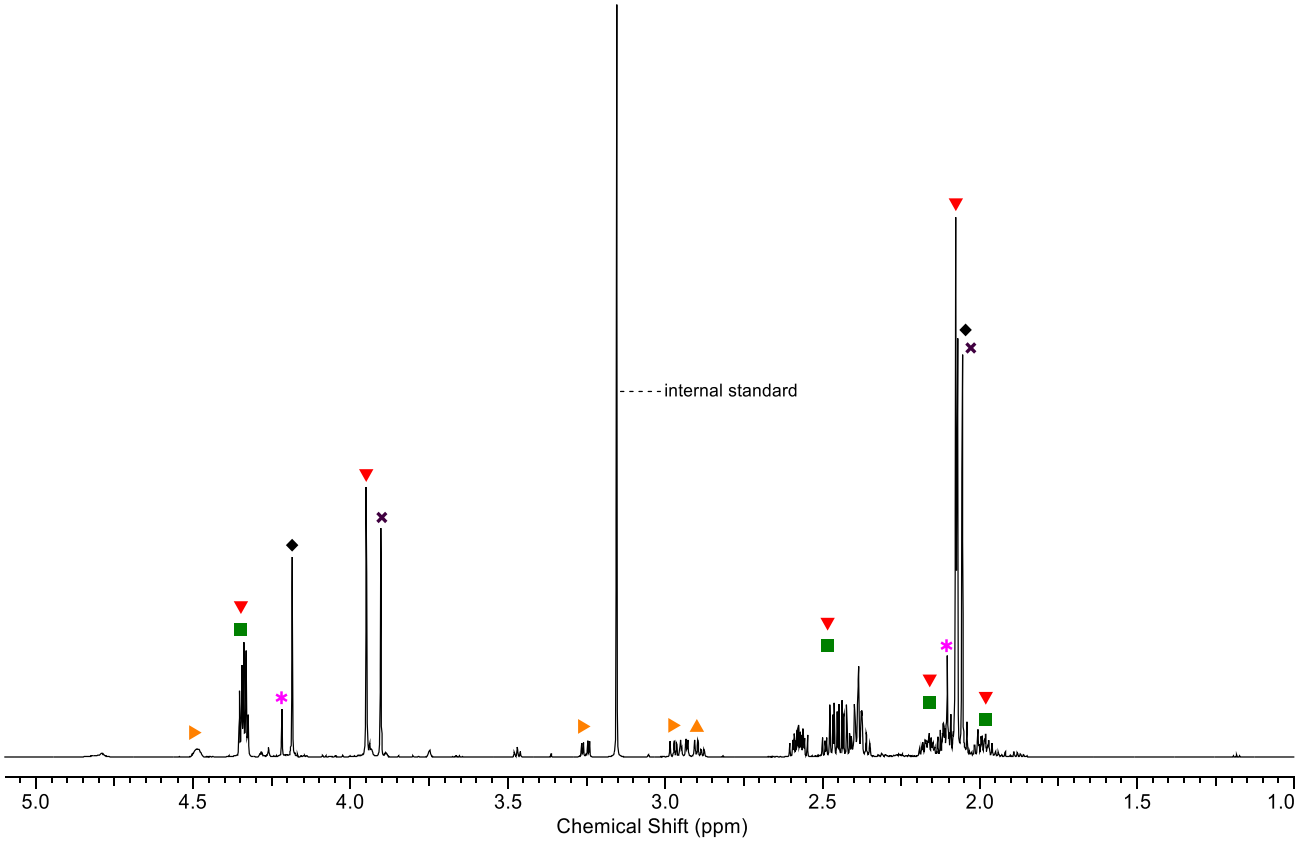


Fig. S148. ^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d, 1.0–5.0 ppm) spectrum to show the reaction of *N*-acetyl glycine nitrile (**Ac-Gly-CN**, 200 mM) with L-glutamine amide (**Gln-NH₂**, 200 mM) and *N*-acetyl-L-cysteine (**Ac-Cys-OH**, 60 mM, 0.3 eq.) with MSM (50 mM) as the internal standard after 24 h at pH 7 and 60 °C. ▶ = *N,N′*-diacetyl-L-cystine (formed by aerial oxidation of **Ac-Cys-OH**), ✖= **Ac-Gly-NH₂** and ✱= **Ac-Gly^{N}-NH₂.**

^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d) *N-Acetylglycylglutaminamide*, **Ac-Gly-Gln-NH₂** (▼) (partial assignment) δ_H 4.35-4.33 (1H, m, Gln-αH-CONH$_2$), 3.95 (2H, s, AcNHC*H*$_2$), 2.08 (3H, s, *H*$_3$C(CO)); *L-glutaminamide*, **Gln-NH₂** (■) (partial assignment): δ_H 4.35-4.33 αH-CONH$_2$; *N-Acetylglycinamide*, **Ac-Gly-NH₂** (✖): δ_H 3.90 (2H, s, C*H*$_2$), 2.05 (3H, s, *H*$_3$C(CO)); *N-(2-amino-2-iminoethyl)acetamide*, **Ac-Gly^{N}-NH₂** (✱): δ_H 4.22 (2H, s, C*H*$_2$), 2.10 (3H, s, *H*$_3$C(CO)).

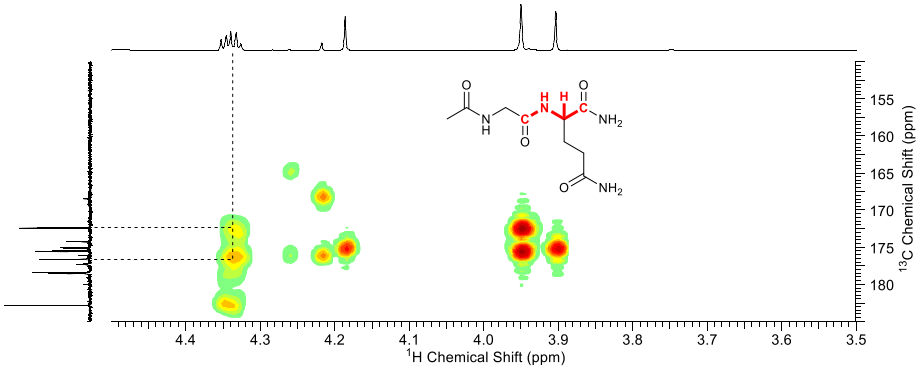


Fig. S149. ^{1}H–^{13}C HMBC (^{1}H: 700 MHz [3.5–4.5 ppm], ^{13}C: 176 MHz [150–185 ppm], H_2O/D_2O 9:1) spectrum showing the diagnostic $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling of the **Gln**-αH-CONH$_2$ in **Ac-Gly-Gln-NH₂** at 4.35-4.33 ppm with two resonances at 177 and 172 ppm, which is characteristic of amide bond formation of **Gln-NH₂**. See Fig. S148 for expanded and labelled ^{1}H NMR spectrum.

Coupling of N-acetylglycine nitrile ***Ac-Gly-CN*** *with L-glutamic acid amide* ***Glu-NH₂*** *at pH 7 and 60 °C*

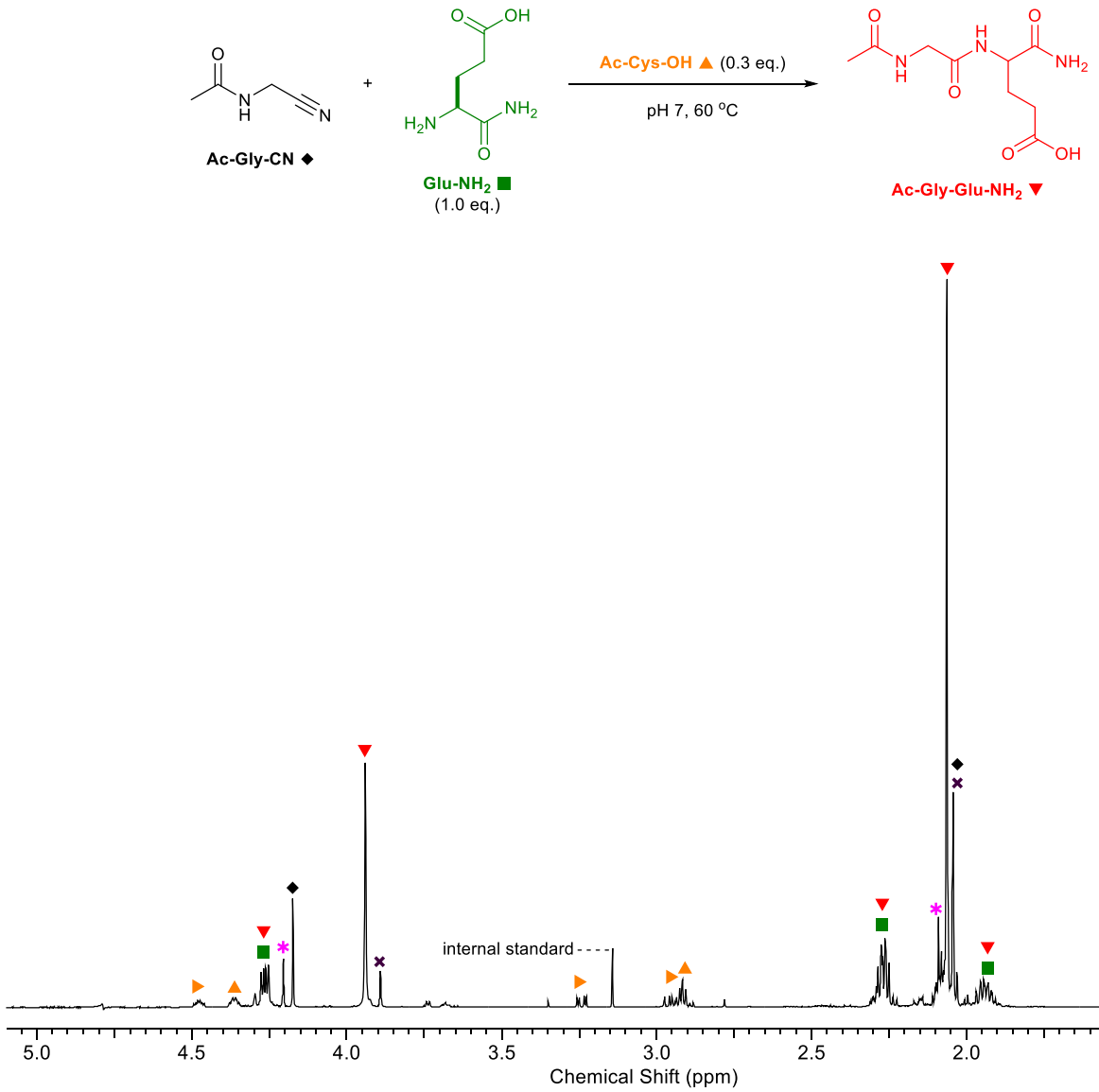


Fig. S150. ^{1}H NMR (600 MHz, H_2O/D_2O 9:1, noesygppr1d, 1.0-5.0 ppm) spectrum to show the reaction of *N*-acetyl glycine nitrile (**Ac-Gly-CN**, 200 mM) with L-glutamic acid amide (**Glu-NH₂**, 200 mM) and *N*-acetyl-L-cysteine (**Ac-Cys-OH**, 60 mM, 0.3 eq.) with MSM (5 mM) as the internal standard after 24 h at pH 7 and 60 °C. ▶ = *N,N'*-diacetyl-L-cystine (formed by aerial oxidation of **Ac-Cys-OH**), ✖= **Ac-Gly-NH₂** and ✱= **Ac-Gly^{N}-NH₂.**

H).

^{1}H NMR (600 MHz, H_2O/D_2O 9:1, noesygppr1d) *N-Acetylglycylglutamic acid amide*, **Ac-Gly-Glu-NH₂** (▼) (partial assignment): δ_H 4.27 (1H, dd, *J* = 9.3, 4.9 Hz, Glu-αH-$CONH_2$), 3.94 (2H, br. s, AcNHCH_2), 2.31-2.22 (2H, m, $CH(CH_2CH_2COOH)CONH_2$), 2.06 (3H, s, $H_3C(CO)$), 1.97-1.91 (2H, m, $CH(CH_2CH_2COOH)CONH_2$); *L-glutamic acid amide*, **Glu-NH₂** (■) (partial assignment): δ_H 4.27 (1H, dd, *J* = 9.3, 4.9 Hz, C*H*(R)), 2.31-2.22 (2H, m, $CH(CH_2CH_2COOH)CONH_2$), 1.97-1.91 (2H, m, $CH(CH_2CH_2COOH)CONH_2$); *N-Acetylglycinamide*, **Ac-Gly-NH₂** (✖) (partial assignment): δ_H 3.89 (2H, s, CH_2), 2.04 (3H, s, $H_3C(CO)$); *N-(2-amino-2-iminoethyl)acetamide*, **Ac-Gly^{N}-NH₂** (✱): δ_H 4.20 (2H, s, CH_2), 2.09 (3H, s, $H_3C(CO)$).

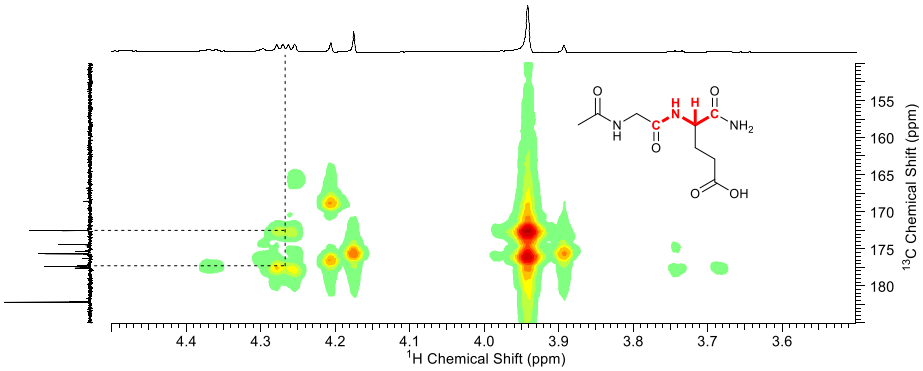


Fig. S151. ^{1}H–^{13}C HMBC (^{1}H: 600 MHz [3.5–4.5 ppm], ^{13}C: 151 MHz [150–185 ppm], H_2O/D_2O 9:1) spectrum showing the diagnostic $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling of the **Glu**-αH-$CONH_2$ in **Ac-Gly-Glu-NH_2** at 4.27 ppm with two resonances at 177 and 173 ppm, which is characteristic of amide bond formation of **Glu-NH_2**. See Fig. S150 for expanded and labelled ^{1}H NMR spectrum.

Coupling of N-acetylglycine nitrile ***Ac-Gly-CN*** *with L-histidinamide* ***His-NH₂*** *at pH 7 and 60 °C*

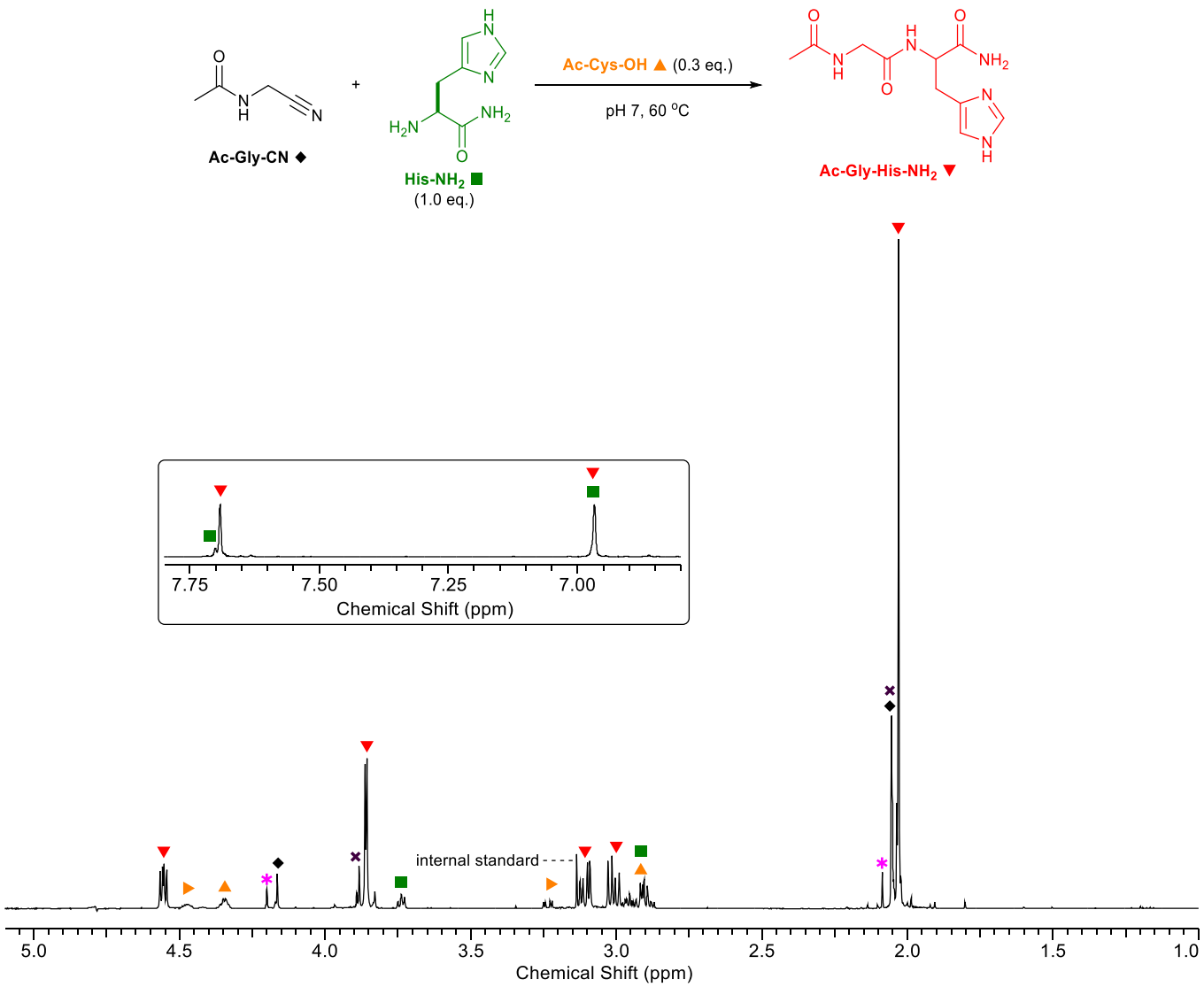


Fig. S152. ^{1}H NMR (600 MHz, H_2O/D_2O 9:1, noesygppr1d, 1.0-5.0 ppm) spectrum to show the reaction of *N*-acetyl glycine nitrile (**Ac-Gly-CN**, 200 mM) with L-histidinamide (**His-NH₂**, 200 mM) and *N*-acetyl-L-cysteine (**Ac-Cys-OH**, 60 mM, 0.3 eq.) with MSM (5 mM) as the internal standard after 24 h at pH 7 and 60 °C. Inset: ^{1}H NMR (600 MHz, H_2O/D_2O 9:1, noesygppr1d, 6.8-7.8 ppm) showing the aromatic CH resonances present in **His** and **Ac-Gly-His-NH₂**. ▶ = *N,N'*-diacetyl-L-cystine (formed by aerial oxidation of **Ac-Cys-OH**), ✖= **Ac-Gly-NH₂** and ✱= **Ac-Gly^N-NH₂.**

^{1}H NMR (600 MHz, H_2O/D_2O 9:1, noesygppr1d) *N-Acetylglycylhistidinamide*, **Ac-Gly-His-NH₂** (▼): δ_H 7.69 (1H, s, Ar*H*), 6.97 (1H, s, Ar*H*), 4.56 (1H, ABX, *J* = 8.4, 5.6 Hz, His-αH-$CONH_2$), 3.88 (1H, AB, *J* = 17.1 Hz, AcNHCH*H*), 3.84 (1H, AB, *J* = 17.1 Hz, AcNHCH*H*), 3.11 (1H, ABX, *J* = 15.0, 5.6 Hz, CHCH*H*Ar), 3.01 (1H, ABX, *J* = 15.0, 8.4 Hz, CHCH*H*Ar), 2.03 (3H, s, $H_3C(CO)$); *L-histidinamide*, **His-NH₂** (■) (partial assignment): δ_H 7.70 (1H, s, Ar*H*), 6.97 (1H, s, Ar*H*), 3.74 (1H, t, *J* = 6.7 Hz, αH-$CONH_2$); *N-Acetylglycinamide*, **Ac-Gly-NH₂** (✖): δ_H 3.88 (2H, s, CH_2), 2.05 (3H, s, $H_3C(CO)$); *N-(2-amino-2-iminoethyl)acetamide*, **Ac-Gly^N-NH₂** (✱): δ_H 4.20 (2H, s, CH_2), 2.09 (3H, s, $H_3C(CO)$).

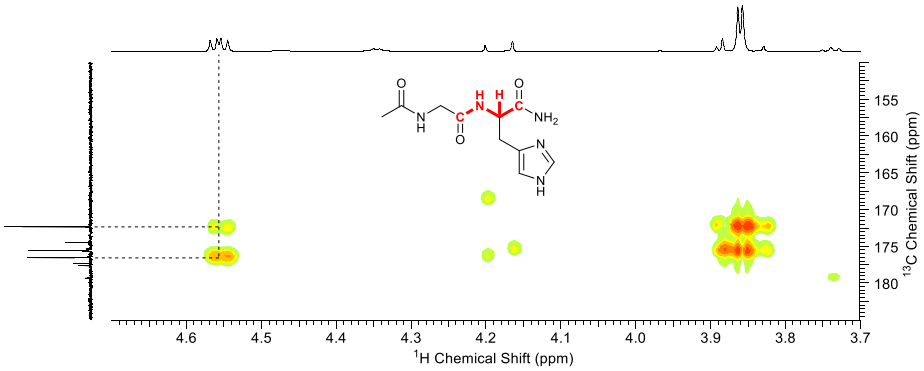


Fig. S153. ^{1}H–^{13}C HMBC (^{1}H: 600 MHz [3.7–4.7 ppm], ^{13}C: 151 MHz [150–185 ppm], H_2O/D_2O 9:1) spectrum showing the diagnostic $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling of the **His**-αH-$CONH_2$ in **Ac-Gly-His-NH_2** at 4.56 ppm with two resonances at 176 and 172 ppm, which is characteristic of amide bond formation of **His-NH_2**. See Fig. S152 for expanded and labelled ^{1}H NMR spectrum.

Coupling of N-acetylglycine nitrile ***Ac-Gly-CN*** *with L-isoleucinamide* ***Ile-NH₂*** *at pH 7 and 60 °C*

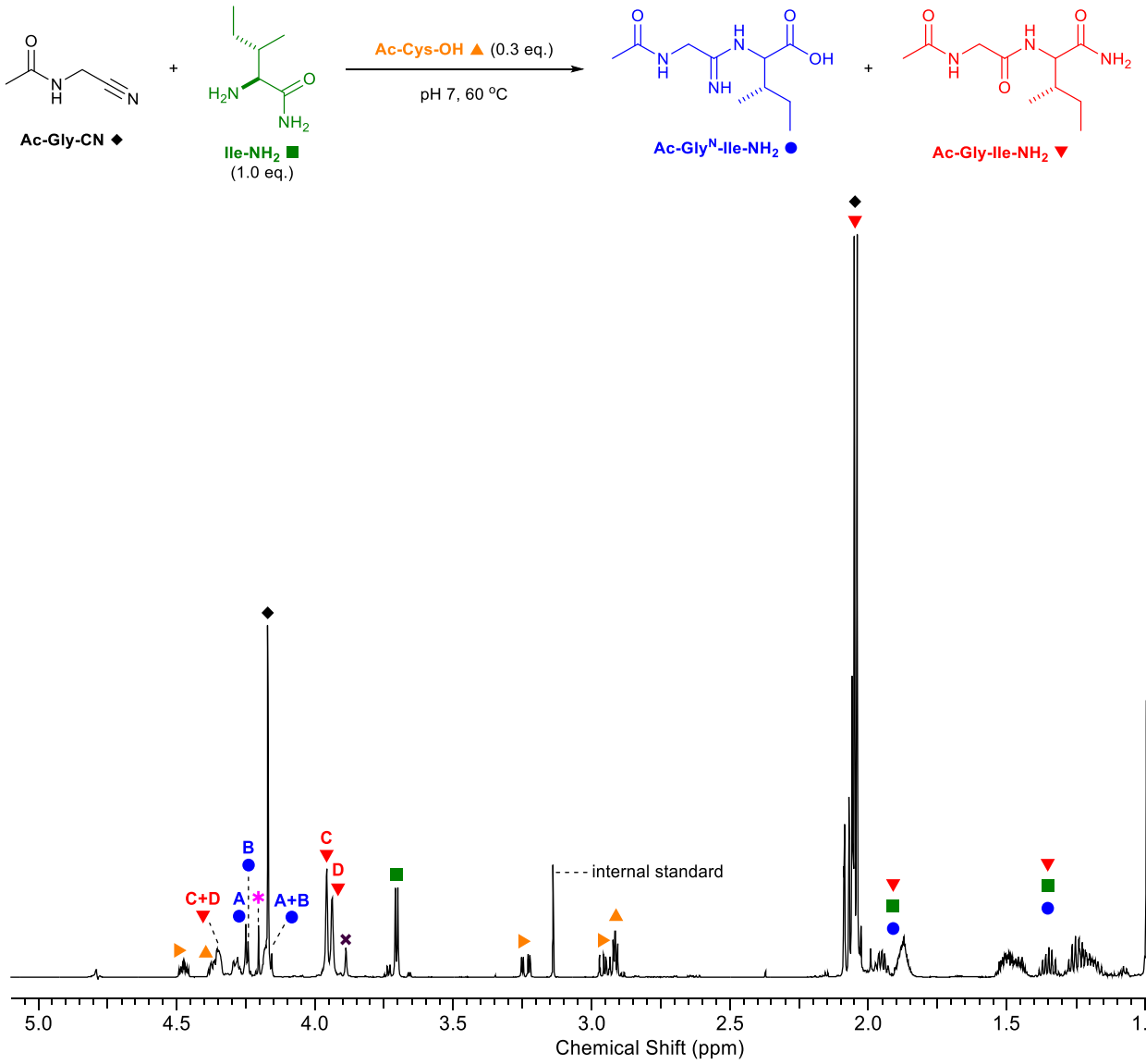


Fig. S154. ^{1}H NMR (600 MHz, H_2O/D_2O 9:1, noesygppr1d, 0.5–5.1 ppm) spectrum to show the reaction of *N*-acetyl glycine nitrile (**Ac-Gly-CN**, 200 mM) with L-isoleucinamide (**Ile-NH₂**, 200 mM) and *N*-acetyl-L-cysteine (**Ac-Cys-OH**, 60 mM, 0.3 eq.) with MSM (5 mM) as the internal standard after 24 h at pH 7 and 60 °C. ▶ = *N,N′*-diacetyl-L-cystine (formed by aerial oxidation of **Ac-Cys-OH**), ✖= **Ac-Gly-NH₂** and ✱= **Ac-Gly^N-NH₂.**

^{1}H NMR (600 MHz, H_2O/D_2O 9:1, noesygppr1d) *(2-acetamido-1-iminoethyl)isoleucinamide*, mixture of diastereoisomers [A:B, 57:43], **Ac-Gly^N-Ile-NH₂** (A, ●) (partial assignment): δ_H 4.25 (2H, s br., AcNHC*H*$_2$), 4.18 (1H, m, Ile-αH-CONH$_2$), (B, ●): δ_H 4.24 (2H, s br., AcNHC*H*$_2$), 4.18 (1H, m br., Ile-αH-CONH$_2$); *N-Acetylglycylisoleucinamide*, mixture of diastereoisomers [C:D, 57:43], **Ac-Gly-Ile-NH₂** (C, ▼) (partial assignment): δ_H 4.35 (1H, m., Ile-αH-CONH$_2$), 3.96 (2H, s br., AcNHC*H*$_2$), (D, ▼) (partial assignment): δ_H 4.35 (1H, s br., Ile-αH-CONH$_2$), 3.94 (2H, s br., AcNHC*H*$_2$); *L-isoleucinamide*, **Ile-NH₂** (■) (partial assignment): δ_H 3.70 (1H, d, *J* = 5.6 Hz, αH-CONH$_2$); *N-Acetylglycinamide*, **Ac-Gly-NH₂** (✖) (partial assignment): δ_H 3.89 (2H, s, C*H*$_2$); *N-(2-amino-2-iminoethyl)acetamide*, **Ac-Gly^N-NH₂** (✱): δ_H 4.20 (2H, s, C*H*$_2$), 2.09 (3H, s, *H*$_3$C(CO)).

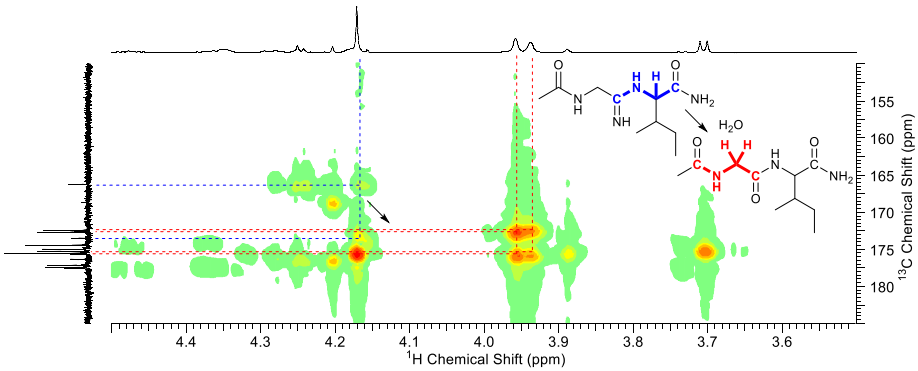


Fig. S155. ^{1}H–^{13}C HMBC (^{1}H: 600 MHz [3.5–4.5 ppm], ^{13}C: 151 MHz [150–185 ppm], H_2O/D_2O 9:1) spectrum showing the diagnostic $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling of the **Ile**-αH-$CONH_2$ in **Ac-Gly^{N}-Ile-NH$_2$** at 4.18 ppm with two resonances at 173 and 166 ppm, which is characteristic of amidine bond formation of **Ile-NH$_2$**, and the diagnostic $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling of the **Gly**-αH in **Ac-Gly-Ile-NH$_2$** at 3.96 and 3.94 ppm with resonances at (176 and 175) and (173 and 172) ppm, which is characteristic of amide bond formation of **Ile-NH$_2$**. See Fig. S154 for expanded and labelled ^{1}H NMR spectrum.

Coupling of N-acetylglycine nitrile ***Ac-Gly-CN*** *with D-leucinamide D-****Leu-NH₂*** *at pH 7 and 60 °C*

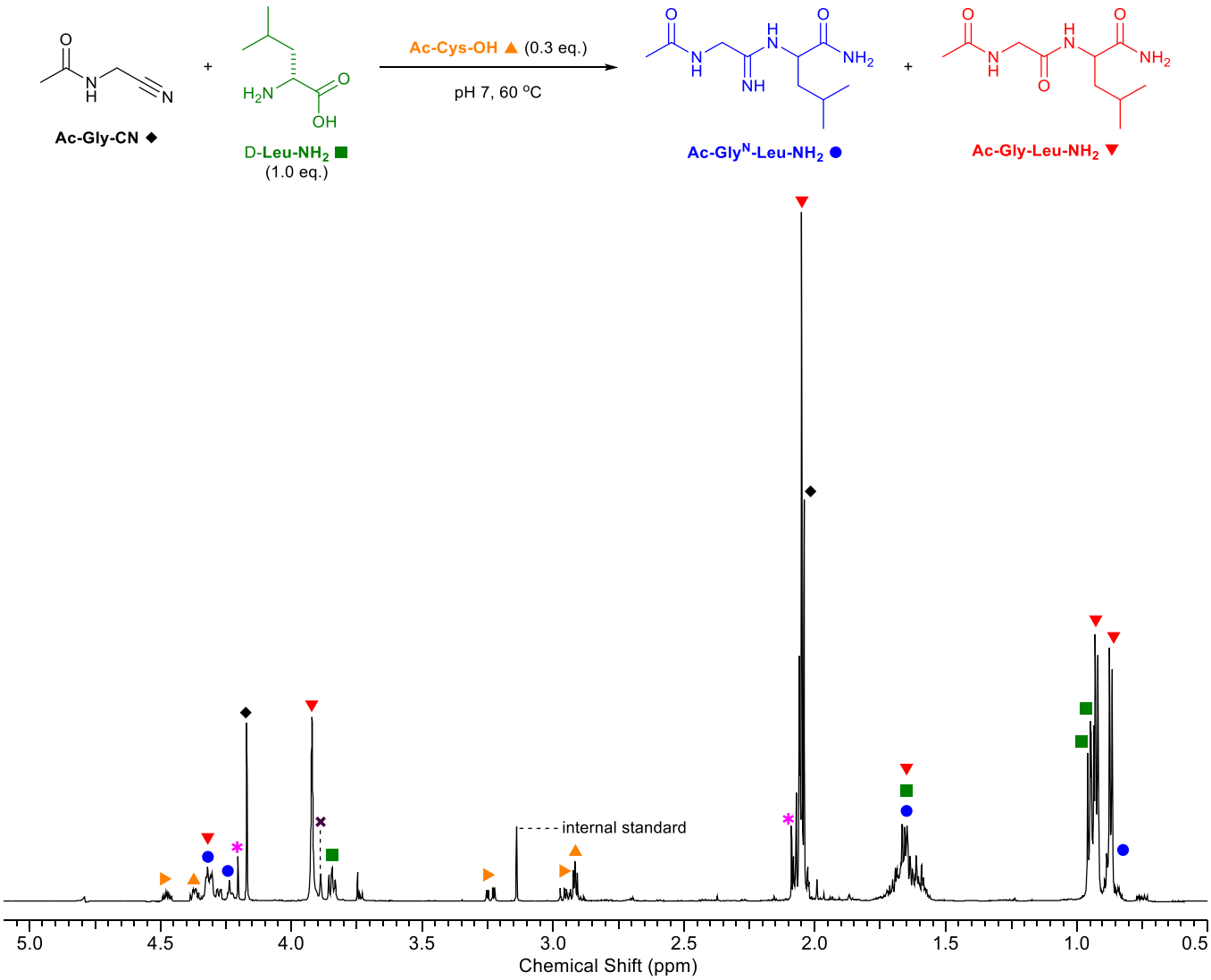


Fig. S156. ^{1}H NMR (600 MHz, H_2O/D_2O 9:1, noesygppr1d, 0.5–5.0 ppm) spectrum to show the reaction of *N*-acetyl glycine nitrile (**Ac-Gly-CN**, 200 mM) with D-leucinamide (D-**Leu-NH₂**, 200 mM) and *N*-acetyl-L-cysteine (**Ac-Cys-OH**, 60 mM, 0.3 eq.) with MSM (5 mM) as the internal standard after 24 h at pH 7 and 60 °C. ▶ = *N,N′*-diacetyl-L-cystine (formed by aerial oxidation of **Ac-Cys-OH**), ✖= **Ac-Gly-NH₂** and ✱= **Ac-Gly^N-NH₂.**

^{1}H NMR (600 MHz, H_2O/D_2O 9:1, noesygppr1d) *(2-acetamido-1-iminoethyl)leucinamide*, **Ac-Gly^N-Leu-NH₂** (●) (partial assignment): δ_H 4.34-4.29 (1H, obs., Leu-αH-$CONH_2$), 4.24 (2H, s, AcNHC*H*$_2$), 1.72-1.56 (3H, m, CHC*H*$_2$CH$(CH_3)_2$; $CHCH_2CH(CH_3)_2$); *N-Acetylglycylleucinamide*, **Ac-Gly-Leu-NH₂** (▼) (partial assignment): δ_H 4.31 (1H, dd, *J* = 10.2, 4.0 Hz, Leu-αH-$CONH_2$), 3.92 (2H, br. s, AcNHC*H*$_2$), 2.05 (3H, s, *H*$_3$C(CO)), 1.72-1.56 (3H, m, CHC*H*$_2$CH$(CH_3)_2$; $CHCH_2CH(CH_3)_2$), 0.92 (3H, d, *J* = 6.2 Hz, C*H*$_3$), 0.87 (3H, d, *J* = 6.2 Hz, C*H*$_3$); *D-leucinamide*, D-**Leu-NH₂** (■) (partial assignment): δ_H 3.84 (1H, t, *J* = 7.1 Hz, αH-$CONH_2$), 1.72-1.56 (3H, m, CHC*H*$_2$CH$(CH_3)_2$; $CHCH_2CH(CH_3)_2$), 0.95 (3H, d, *J* = 6.2 Hz, C*H*$_3$), 0.94 (3H, d, *J* = 6.2 Hz, C*H*$_3$); *N-Acetylglycinamide*, **Ac-Gly-NH₂** (✖) (partial assignment): δ_H 3.89 (2H, s, C*H*$_2$); *N-(2-amino-2-iminoethyl)acetamide*, **Ac-Gly^N-NH₂** (✱): δ_H 4.20 (2H, s, C*H*$_2$), 2.09 (3H, s, *H*$_3$C(CO)).

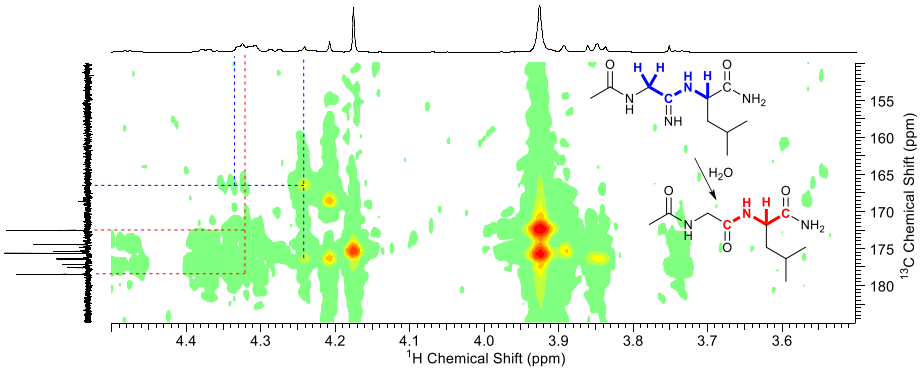
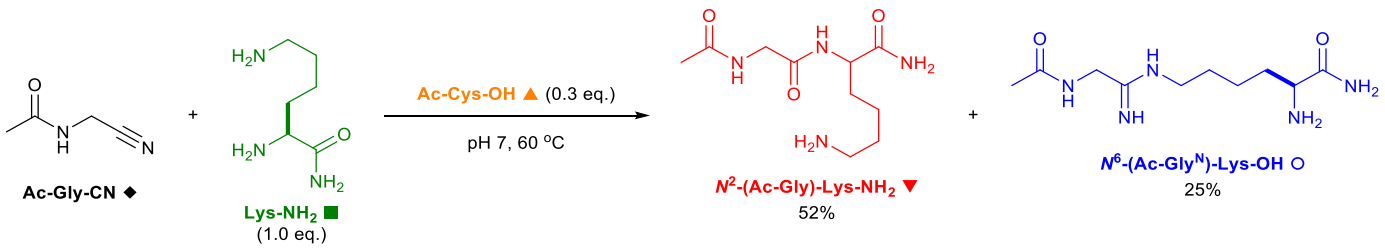


Fig. S157. ^{1}H–^{13}C HMBC (^{1}H: 600 MHz [3.5–4.5 ppm], ^{13}C: 151 MHz [150-185 ppm], H_2O/D_2O 9:1) spectrum showing the diagnostic $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling of the **Leu**-αH-$CONH_2$ and glycyl CH_2 in **Ac-Gly^{N}-Leu-NH$_2$** at 4.34-4.29 and 4.24 ppm with a resonance at 166 ppm, which is characteristic of amidine bond formation of D-**Leu-NH$_2$**, and the diagnostic $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling of **Leu**-αH-$CONH_2$ in **Ac-Gly-Leu-NH$_2$** at 4.34-4.29 ppm with two resonances at 178 and 173 ppm, which is characteristic of amide bond formation of D-**Leu-NH$_2$**. See Fig. S156 for expanded and labelled ^{1}H NMR spectrum.

Coupling of N-acetylglycine nitrile ***Ac-Gly-CN*** *with L-lysinamide* ***Lys-NH₂*** *at pH 7 and 60 ℃*



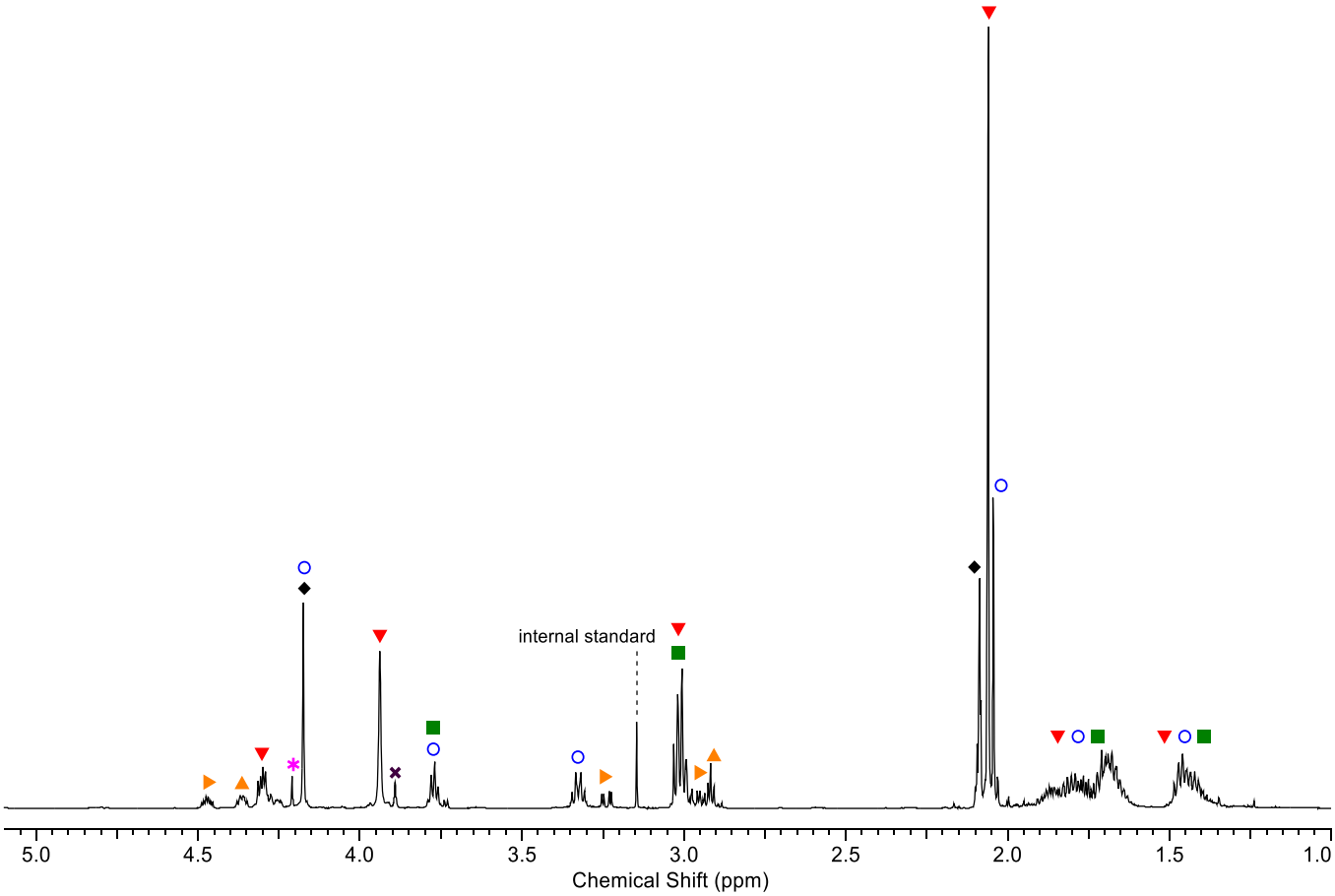


Fig. S158. ^{1}H NMR (600 MHz, H_2O/D_2O 9:1, noesygppr1d, 1.0-5.1 ppm) spectrum to show the reaction of *N*-acetyl glycine nitrile (**Ac-Gly-CN**, 200 mM) with L-lysinamide (**Lys-NH₂**, 200 mM) and *N*-acetyl-L-cysteine (**Ac-Cys-OH**, 60 mM, 0.3 eq.) with MSM (5 mM) as the internal standard after 24 h at pH 7 and 60 °C. ▶ = *N,N′*-diacetyl-L-cystine (formed by aerial oxidation of **Ac-Cys-OH**), ✖= **Ac-Gly-NH₂** and ✱= **Ac-Gly^{N}-NH₂.**

^{1}H NMR (600 MHz, H_2O/D_2O 9:1, noesygppr1d) N^{6}*-(2-acetamido-1-iminoethyl)lysinamide*, ***N*^{6}-(Ac-Gly^{N})-Lys-NH₂** (○): δ_H 4.18 (2H, s, *N*^{6}-AcNHC*H*$_2$), 3.79-3.75 (1H, m, lysyl-αH-CONH$_2$), 3.35-3.31 (2H, m, *N*^{6}-C*H*$_2$CH$_2$), 2.04 (3H, s, *N*^{6}-*H*$_3$C(CO)); N^{2}*-Acetylglycyllysinamide*, ***N*^{2}-(Ac-Gly)-Lys-NH₂** (▼): δ_H 4.30 (1H, dd, *J* = 9.3, 4.9 Hz, lysyl-αH-CONH$_2$), 3.94 (2H, *N*^{2}-AcNHC*H*$_2$), 3.03-2.99 (2H, m, *N*^{6}-NH$_2$C*H*$_2$CH$_2$), 2.06 (3H, s, *N*^{2}-*H*$_3$C(CO)); *L-lysinamide*, **Lys-NH₂** (■): δ_H 3.79-3.75 (1H, m, lysyl-αH-CONH$_2$), 3.03-2.99 (2H, m, *N*^{6}-NH$_2$C*H*$_2$CH$_2$); *N-Acetylglycinamide*, **Ac-Gly-NH₂** (✖): δ_H 3.89 (2H, s, C*H*$_2$); *N-(2-amino-2-iminoethyl)acetamide*, **Ac-Gly^{N}-NH₂** (✱): δ_H 4.21 (2H, s, C*H*$_2$).

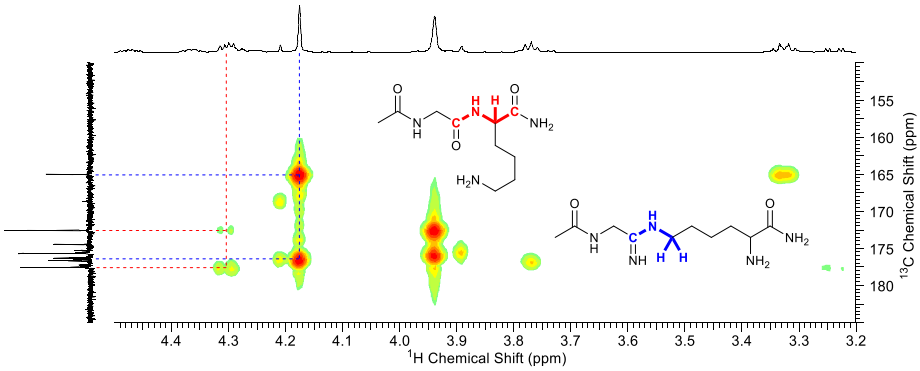


Fig. S159. ^{1}H–^{13}C HMBC (^{1}H: 600 MHz [3.2–4.5 ppm], ^{13}C: 151 MHz [150–185 ppm], H_2O/D_2O 9:1) spectrum showing the diagnostic $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling of the **Lys**-N^{6}-CH_2 in ***N*^{6}-(Ac-Gly^{N})-Lys-NH$_2$** at 4.18 ppm with two resonances at 177 and 165 ppm, which is characteristic of amidine bond formation of **Lys-NH$_2$** and the diagnostic $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling of the **Lys**-αH-$CONH_2$ in ***N*^{2}-(Ac-Gly)-Lys-NH$_2$** at 4.30 ppm with two resonances at 178 and 173 ppm, which is characteristic of amide bond formation of **Lys-NH$_2$**. See Fig. S158 for expanded and labelled ^{1}H NMR spectrum.

Coupling of N-acetylglycine nitrile ***Ac-Gly-CN*** *with L-methioninamide* ***Met-NH₂*** *at pH 7 and 60 °C*

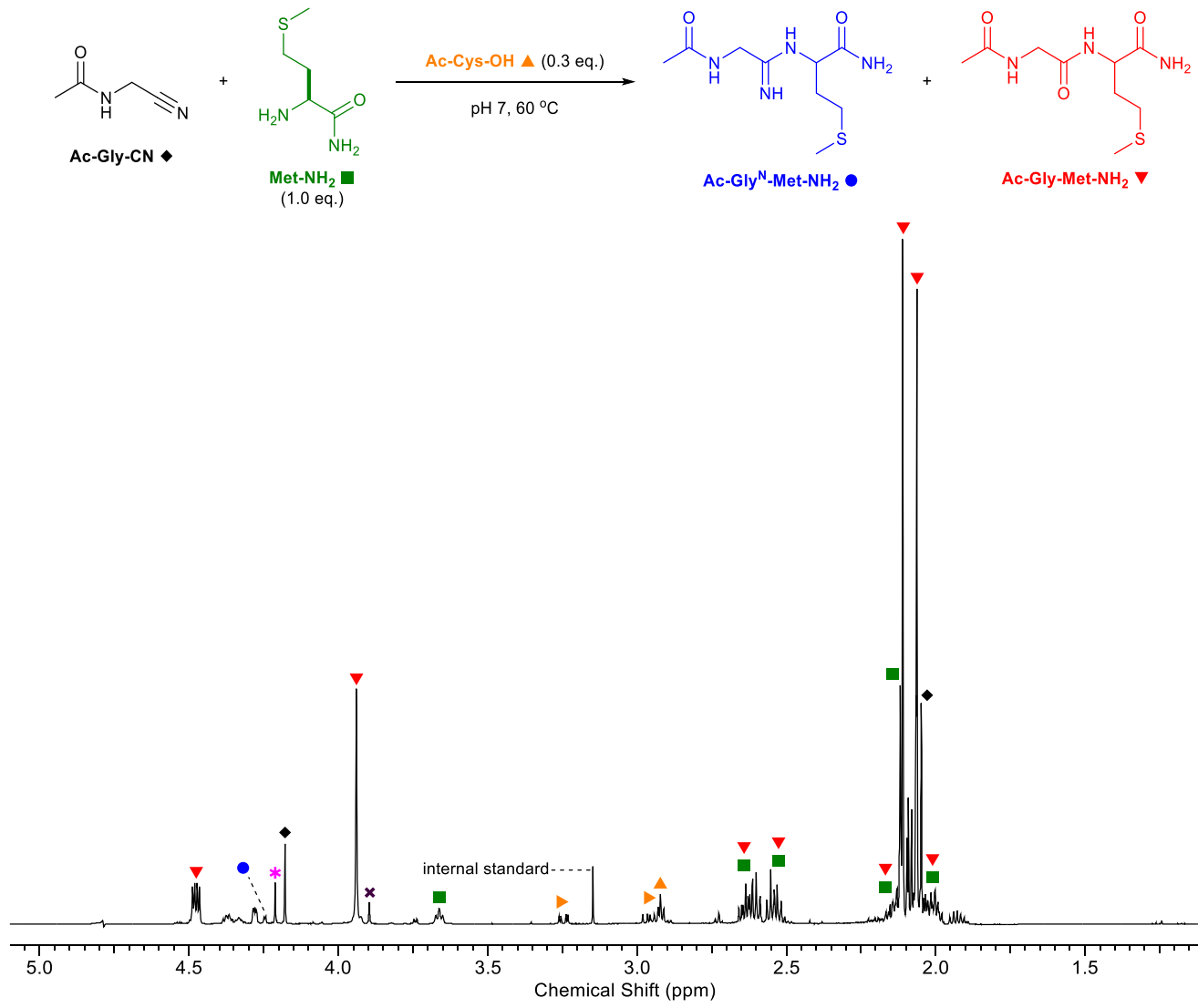


Fig. S160. ^{1}H NMR (600 MHz, H_2O/D_2O 9:1, noesygppr1d, 1.0-5.0 ppm) spectrum to show the reaction of *N*-acetyl glycine nitrile (**Ac-Gly-CN**, 200 mM) with L-methioninamide (**Met-NH₂**, 200 mM) and *N*-acetyl-L-cysteine (**Ac-Cys-OH**, 60 mM, 0.3 eq.) with MSM (5 mM) as the internal standard after 24 h at pH 7 and 60 °C. ▶ = *N,N'*-diacetyl-L-cystine (formed by aerial oxidation of **Ac-Cys-OH**), ✖= **Ac-Gly-NH₂** and ✱= **Ac-Gly^N-NH₂.**

^{1}H NMR (600 MHz, H_2O/D_2O 9:1, noesygppr1d) *(2-acetamido-1-iminoethyl)methioninamide*, **Ac-Gly^N-Met-NH₂** (●) (partial assignment): δ_H 4.25 (2H, app. d., AcNHC*H*$_2$); *N-Acetylglycylmethioninamide*, **Ac-Gly-Met-NH₂** (▼) (partial assignment): δ_H 4.48 (1H, dd, *J* = 9.7, 4.6 Hz, Met-αH-CONH$_2$), 3.94 (2H, br. s., AcNHC*H*$_2$), 2.66-2.59 (1H, m, CH*H*SCH$_3$), 2.57-2.51 (1H, m, CH*H*SCH$_3$), 2.17-1.98 (2H, m., CHC*H*$_2$), 2.11 (3H, s, SC*H*$_3$), 2.06 (3H, s, *H*$_3$C(CO)); *L-methioninamide*, **Met-NH₂** (■): δ_H 3.66 (1H, t, *J* = 6.6 Hz, αH-CONH$_2$), 2.66-2.59 (1H, m, CH*H*SCH$_3$), 2.57-2.51 (1H, m, CH*H*SCH$_3$), 2.17-1.98 (1H, br. m., CHCH*H*), 2.12 (3H, s, SC*H*$_3$), 1.95-1.89 (1H, m, CHCH*H*); *N-Acetylglycinamide*, **Ac-Gly-NH₂** (✖) (partial assignment): δ_H 3.90 (2H, s, C*H*$_2$); *N-(2-amino-2-iminoethyl)acetamide*, **Ac-Gly^N-NH₂** (✱) (partial assignment): δ_H 4.21 (2H, s, C*H*$_2$).

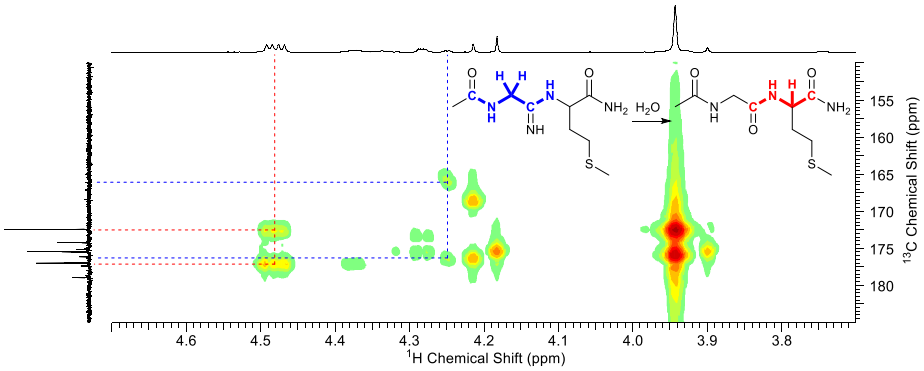


Fig. S161. ^{1}H–^{13}C HMBC (^{1}H: 600 MHz [3.7–4.7 ppm], ^{13}C: 151 MHz [150–185 ppm], H_2O/D_2O 9:1) spectrum showing the diagnostic $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling of the **Met**-αH-$CONH_2$ in **Ac-Gly^{N}-Met-NH$_2$** at 4.25 ppm with two resonances at 176 and 166 ppm, which is characteristic of amidine bond formation of **Met-NH$_2$**, and the diagnostic $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling of the **Met**-αH-$CONH_2$ in **Ac-Gly-Met-NH$_2$** at 4.48 ppm with two resonances at 177 and 172 ppm, which is characteristic of amide bond formation of **Met-NH$_2$**. See Fig. S160 for expanded and labelled ^{1}H NMR spectrum.

Coupling of N-acetylglycine nitrile ***Ac-Gly-CN*** *with L-phenylalaninamide* ***Phe-NH₂*** *at pH 7 and 60 °C*

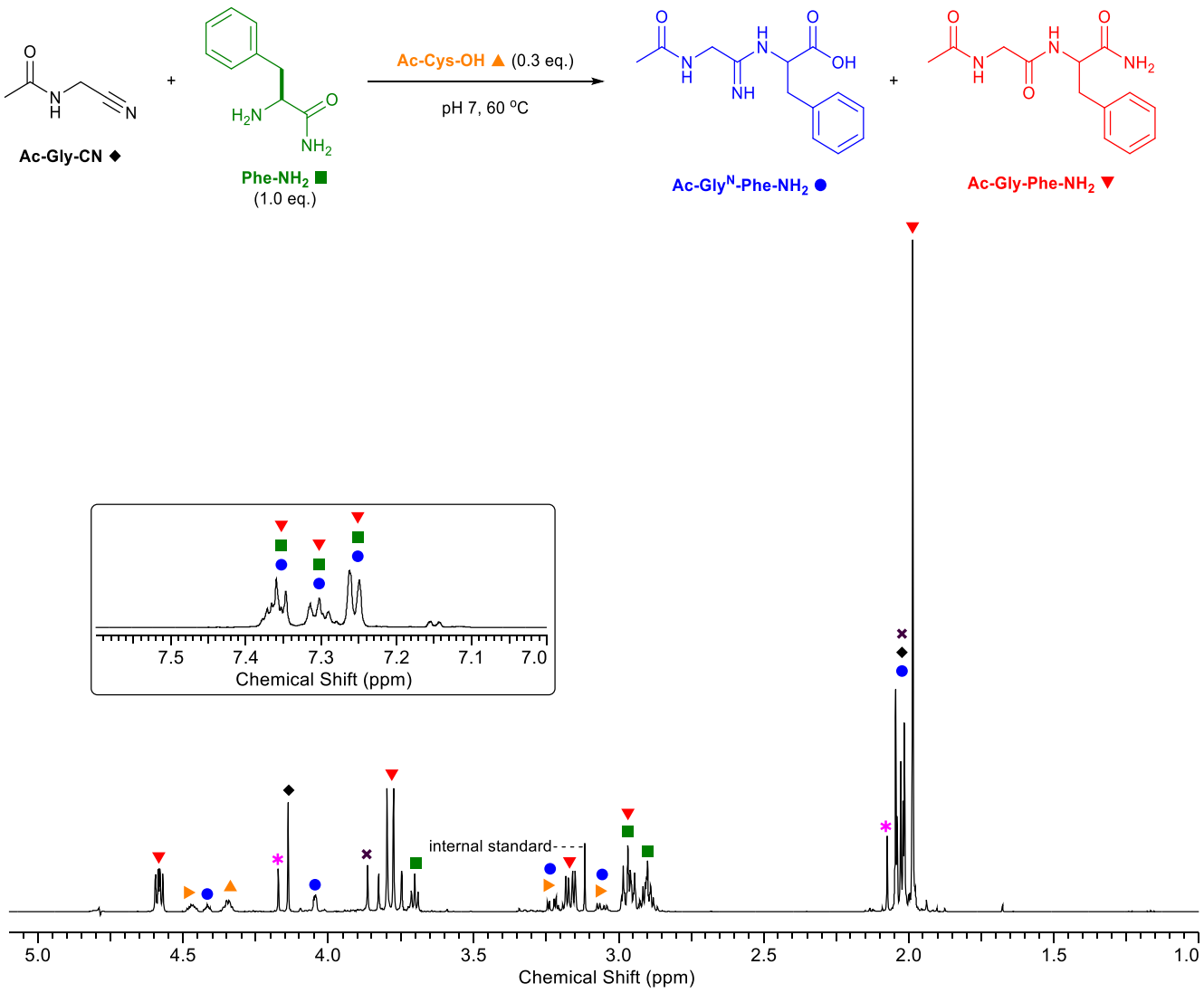


Fig. S162. ^{1}H NMR (600 MHz, H_2O/D_2O 9:1, noesygppr1d, 1.0–5.0 ppm) spectrum to show the reaction of *N*-acetyl glycine nitrile (**Ac-Gly-CN**, 200 mM) with L-phenylalaninamide (**Phe-NH₂**, 200 mM) and *N*-acetyl-L-cysteine (**Ac-Cys-OH**, 60 mM, 0.3 eq.) with MSM (5 mM) as the internal standard after 24 h at pH 7 and 60 °C. Inset: ^{1}H NMR (600 MHz, H_2O/D_2O 9:1, noesygppr1d, 7.0-7.6 ppm) showing the aromatic CH resonances of **Phe-NH₂**, **Ac-Gly^N-Phe-NH₂** and **Ac-Gly-Phe-NH₂**. ▶ = *N,N'*-diacetyl-L-cystine (formed by aerial oxidation of **Ac-Cys-OH**), ✖= **Ac-Gly-NH₂** and ✱= **Ac-Gly^N-NH₂**.

^{1}H NMR (600 MHz, H_2O/D_2O 9:1, noesygppr1d) *(2-acetamido-1-iminoethyl)phenylalaninamide*, **Ac-Gly^N-Phe-NH₂** (●) (partial assignment): δ_H 4.42 (1H, app. t, *J* = 5.3 Hz, Phe-αH-CONH₂), 4.05-4.04 (2H, m, AcNHC*H*₂), 3.23 (1H, ABX, *J* = 14.0, 4.1 Hz, CHCH*H*); *N-Acetylglycylphenylalaninamide*, **Ac-Gly-Phe-NH₂** (▼) (partial assignment): δ_H 4.58 (1H, ABX, *J* = 8.9, 5.8 Hz, Phe-αH-CONH₂), 3.81 (1H, AB, *J* = 17.1 Hz, AcNHCH*H*), 3.76 (1H, AB, *J* = 17.1 Hz, AcNHCH*H*), 3.17 (1H, dd, *J* = 14.0, 5.8 Hz, CHCH*H*Ar), 2.99-2.94 (1H, m, CHCH*H*Ar), 1.99 (3H, s, *H*$_3$C(CO)); *L-phenylalaninamide*, **Phe-NH₂** (■) (partial assignment): 3.70 (1H, t, *J* = 6.9 Hz, αH-CONH₂), 2.99-2.94 (1H, m, CHCH*H*Ar), 2.93-2.87 (1H, m, CHCH*H*Ar); *N-Acetylglycinamide*, **Ac-Gly-NH₂** (✖) (partial assignment): δ_H 3.86 (2H, s, C*H*₂); *N-(2-amino-2-iminoethyl)acetamide*, **Ac-Gly^N-NH₂** (✱): δ_H 4.17 (2H, s, C*H*₂), 2.07 (3H, s, *H*$_3$C(CO)).

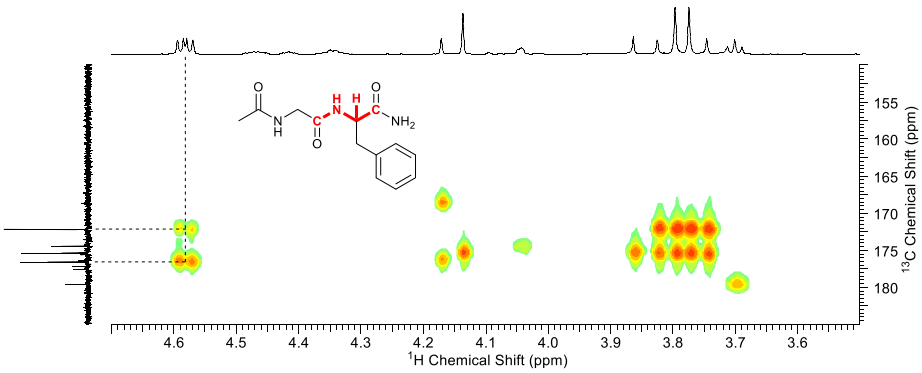


Fig. S163. ^{1}H–^{13}C HMBC (^{1}H: 600 MHz [3.5–4.7 ppm], ^{13}C: 151 MHz [150–185 ppm], H_2O/D_2O 9:1) spectrum showing the diagnostic $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling of the **Phe**-αH-$CONH_2$ of **Ac-Gly-Phe-NH_2** at 4.58 ppm with two resonances at 177 and 172 ppm, which is characteristic of amide bond formation of **Phe-NH_2**. See Fig. S162 for expanded and labelled ^{1}H NMR spectrum.

Coupling of N-acetylglycine nitrile ***Ac-Gly-CN*** *with L-prolinamide* ***Pro-NH₂*** *at pH 7 and 60 °C*

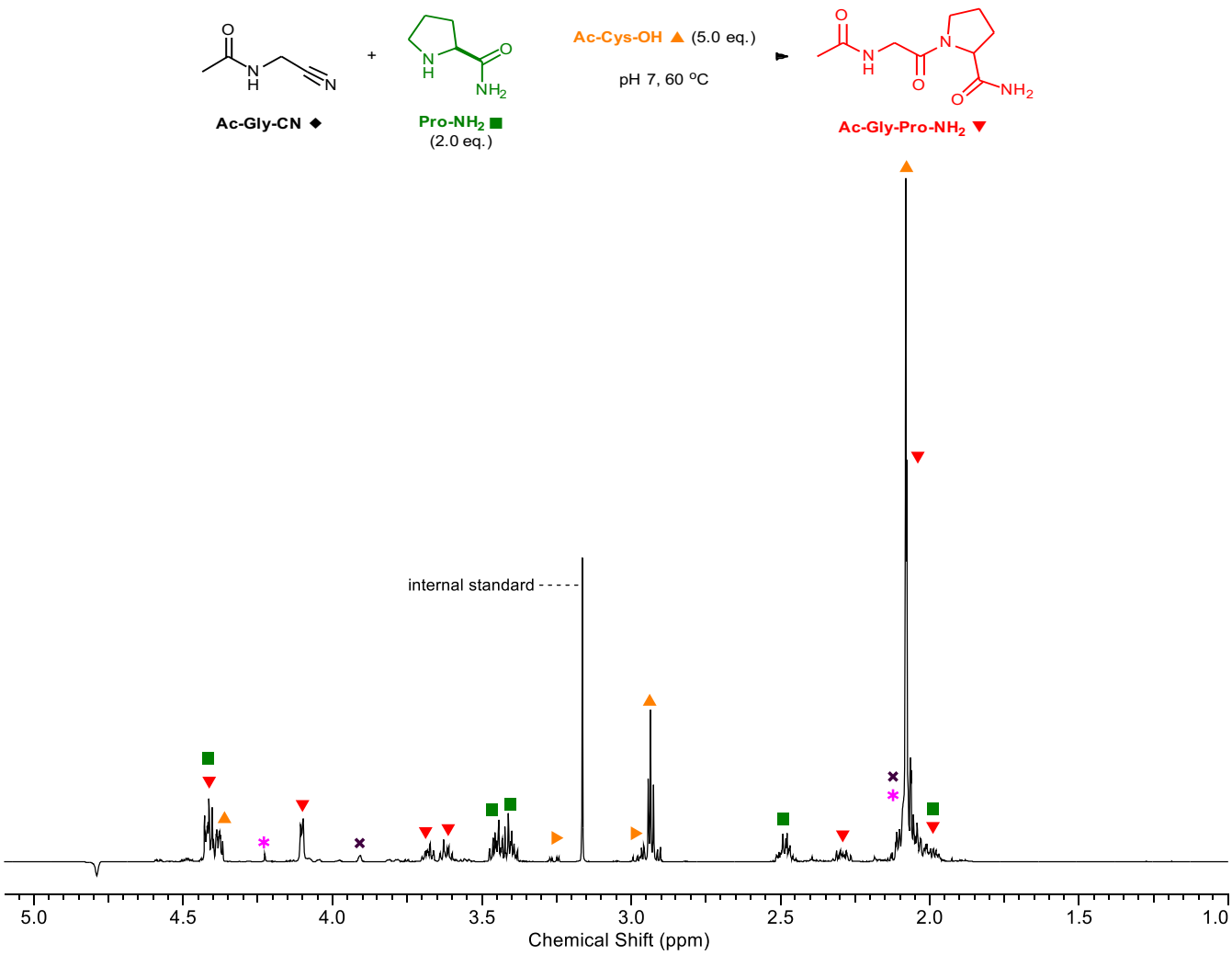


Fig. S164. ^{1}H NMR (600 MHz, H_2O/D_2O 9:1, noesygppr1d, 1.0–5.0 ppm) spectrum to show the reaction of *N*-acetyl glycine nitrile (**Ac-Gly-CN**, 200 mM) with L-prolinamide (**Pro-NH₂**, 400 mM) and *N*-acetyl-L-cysteine (**Ac-Cys-OH**, 300 mM, 5.0 eq.) with MSM (50 mM) as the internal standard after 24 h at pH 7 and 60 °C. ▶ = *N,N'*-diacetyl-L-cystine (formed by aerial oxidation of **Ac-Cys-OH**), ✖= **Ac-Gly-NH₂** and ✱= **Ac-Gly^{N}-NH₂.**

^{1}H NMR (600 MHz, H_2O/D_2O 9:1, noesygppr1d) *N-Acetylglycylprolinamide*, **Ac-Gly-Pro-NH₂** (▼) (partial assignment): δ_H 4.43-4.40 (1H, m, Pro-αH-$CONH_2$), 4.10 (2H, app. d, AcNHC*H*$_2$), 3.70-3.66 (1H, m, NC*H*CH_2CH_2), 3.64-3.60 (1H, m, NC*H*CH_2CH_2), 2.08 (3H, s, H_3C(CO)); *L-prolinamide*, **Pro-NH₂** (■) (partial assignment): δ_H 4.43-4.40 (1H, m, αH-$CONH_2$), 3.47-3.39 (2H, m, HNC*H*$_2CH_2CH_2$); *N-Acetylglycinamide*, **Ac-Gly-NH₂** (✖) (partial assignment): δ_H 3.91 (2H, s, C*H*$_2$); *N-(2-amino-2-iminoethyl)acetamide*, **Ac-Gly^{N}-NH₂** (✱) (partial assignment): δ_H 4.23 (2H, s, C*H*$_2$).

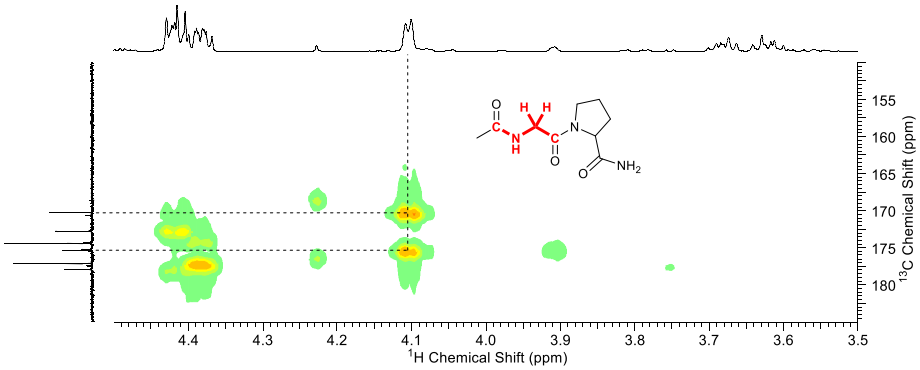


Fig. S165. ^{1}H–^{13}C HMBC (^{1}H: 600 MHz [3.5-4.5 ppm], ^{13}C: 151 MHz [150–185 ppm], H_2O/D_2O 9:1) spectrum showing the diagnostic $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling of the **Gly**-αH in **Ac-Gly-Pro-NH$_2$** at 4.10 ppm with two resonances at 175 and 170 ppm, which is characteristic of amide bond formation of **Pro-NH$_2$**. See Fig. S164 for expanded and labelled ^{1}H NMR spectrum.

Coupling of N-acetylglycine nitrile ***Ac-Gly-CN*** *with L-serinamide* ***Ser-NH₂*** *at pH 7 and 60 °C*

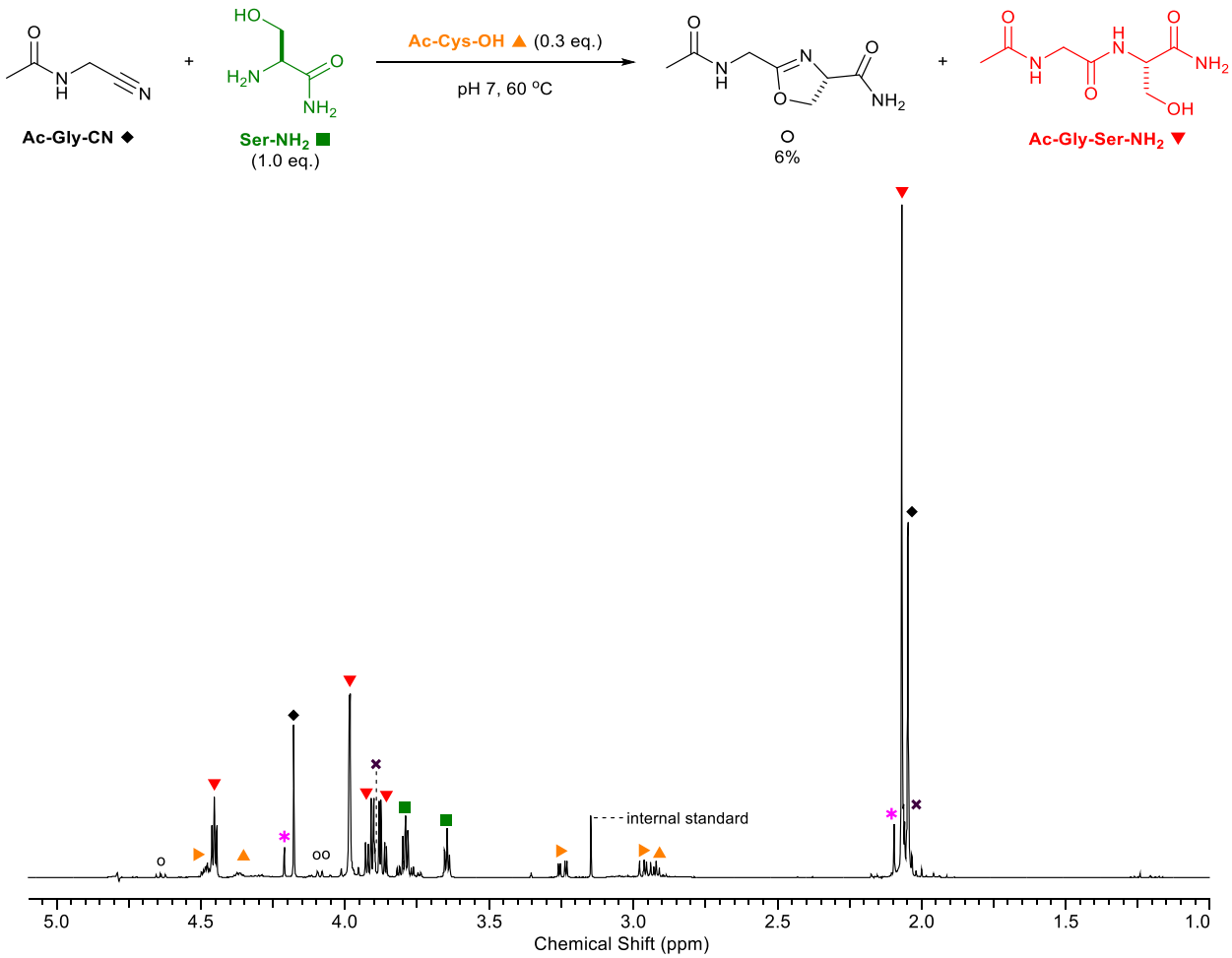


Fig. S166. ^{1}H NMR (600 MHz, H_2O/D_2O 9:1, noesygppr1d, 1.0–5.0 ppm) spectrum to show the reaction of *N*-acetyl glycine nitrile (**Ac-Gly-CN**, 200 mM) with L-serinamide (**Ser-NH₂**, 200 mM) and *N*-acetyl-L-cysteine (**Ac-Cys-OH**, 60 mM, 0.3 eq.) with MSM (5 mM) as the internal standard after 24 h at pH 7 and 60 °C. ▶ = *N,N′*-diacetyl-L-cystine (formed by aerial oxidation of **Ac-Cys-OH**), ✖= **Ac-Gly-NH₂** and ✱= **Ac-Gly^N-NH₂.**

^{1}H NMR (600 MHz, H_2O/D_2O 9:1, noesygppr1d) *N-Acetylglycylserinamide*, **Ac-Gly-Ser-NH₂** (▼): δ_H 4.45 (1H, app. t, *J* = 5.0 Hz, Ser-αH-CONH₂), 4.00 (1H, AB, *J* = 17.2 Hz, AcNHCH*H*), 3.97 (1H, AB, *J* = 17.2 Hz, AcNHCH*H*), 3.91 (1H, dd, *J* = 11.6, 5.5 Hz, CHCH*H*OH), 3.87 (1H, dd, *J* = 11.6, 4.4 Hz, CHCH*H*OH), 2.07 (3H, s, *H*$_3$C(CO)); *2-(acetamidomethyl)-4,5-dihydro-4-carboxamide* (O) (partial assignment): δ_H 4.64 (1H, dd, *J* = 10.8, 8.9 Hz, C*H*CONH₂), 4.11 (1H, AB, *J* = 16.2 Hz, AcNHCH*H*), 4.07 (1H, AB, *J* = 16.2 Hz, AcNHCH*H*); *L-serinamide*, **Ser-NH₂** (■) (partial assignment): δ_H 3.82-3.76 (2H, m, CHC*H*$_2$OH), 3.65 (1H, t, *J* = 5.0 Hz, αH-CONH₂); *N-Acetylglycinamide*, **Ac-Gly-NH₂** (✖): δ_H 3.90 (2H, s, C*H*$_2$); *N-(2-amino-2-iminoethyl)acetamide*, **Ac-Gly^N-NH₂** (✱): δ_H 4.21 (2H, s, C*H*$_2$), 2.10 (3H, s, *H*$_3$C(CO)).

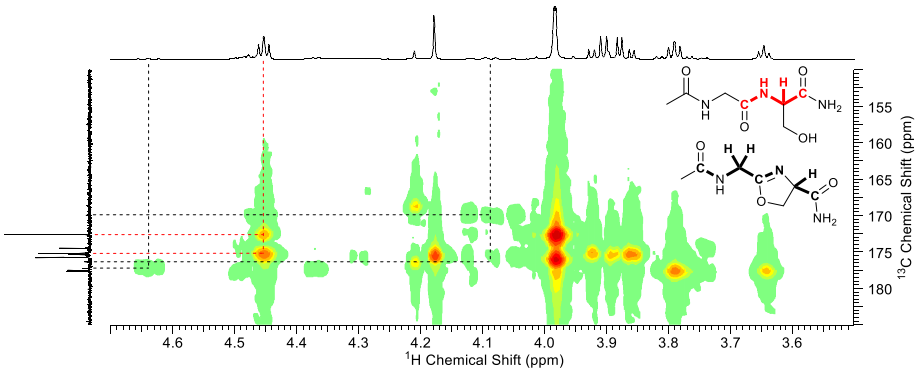


Fig. S167. ^{1}H–^{13}C HMBC (^{1}H: 600 MHz [3.5–4.7 ppm], ^{13}C: 151 MHz [150–185 ppm], H_2O/D_2O 9:1) spectrum showing the diagnostic $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling of the **Ser**-αH-$CONH_2$ in **Ac-Gly-Ser-NH_2** at 4.45 ppm with two resonances at 175 and 173 ppm, which is characteristic of amide bond formation of **Ser-NH_2**, and the diagnostic $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling of the **Gly**-αH_2 of *2-(acetamidomethyl)-4,5-dihydrooxazole-4-carboxamide* at 4.11 and 4.07 ppm with two resonances at 176 and 169 ppm, and a resonance at 4.64 ppm (C4–H) showing a $^{2}J_{CH}$ to 177 ppm. See Fig. S166 for expanded and labelled ^{1}H NMR spectrum.

Coupling of N-acetylglycine nitrile ***Ac-Gly-CN*** *with L-threoninamide* ***Thr-NH₂*** *at pH 7 and 60 °C*

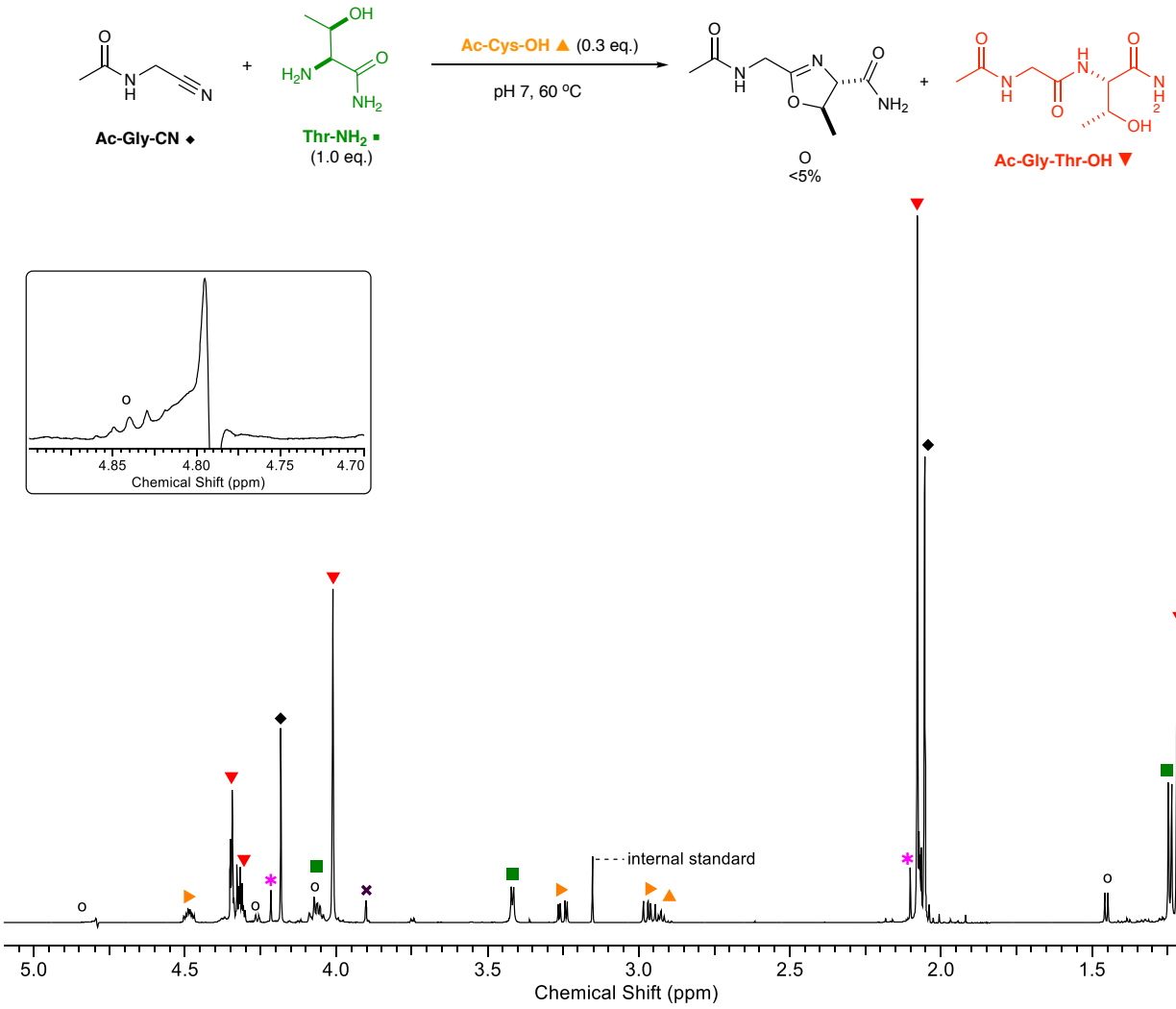


Fig. S168. ^{1}H NMR (600 MHz, H_2O/D_2O 9:1, noesygppr1d, 1.0–5.1 ppm) spectrum to show the reaction of *N*-acetyl glycine nitrile (**Ac-Gly-CN**, 200 mM) with L-threoninamide (**Thr-NH₂**, 200 mM) and *N*-acetyl-L-cysteine (**Ac-Cys-OH**, 60 mM, 0.3 eq.) with MSM (5 mM) as the internal standard after 24 h at pH 7 and 60 °C. ▶ = *N,N′*-diacetyl-L-cystine (formed by aerial oxidation of **Ac-Cys-OH**), ✖= **Ac-Gly-NH₂** and ✱= **Ac-Gly^{N}-NH₂.**

^{1}H NMR (600 MHz, H_2O/D_2O 9:1, noesygppr1d) *N-Acetylglycylthreoninamide*, **Ac-Gly-Thr-NH₂** (▼): δ_H 4.35 (1H, d, *J* = 3.4 Hz, Thr-αH-CONH$_2$), 4.33-4.31 (1H, m, H_3CC*H*OH), 4.01 (2H, br. s., AcNHC*H*$_2$), 2.08 (3H, s, *H*$_3$C(CO)), 1.21 (3H, d, *J* = 6.5 Hz, *H*$_3$CCHOH); *2-(acetamidomethyl)-5-methyl-4,5-dihydrooxazole-4-carboxamide* (O) (partial assignment): δ_H 4.86-4.82 (1H, m, OC*H*CH$_3$), 4.26 (1H, d, *J* = 6.7 Hz, C*H*CONH$_2$), 4.09-4.04 (2H, m, AcNHC*H*$_2$), 1.45 (3H, d, *J* = 6.3 Hz, OCHC*H*$_3$); *L-threoninamide*, **Thr-NH₂** (■): δ_H 4.09-4.04 (1H, m, H_3CC*H*OH), 3.42 (1H, d, *J* = 4.5 Hz, αH-CONH$_2$), 1.24 (3H, d, *J* = 6.5 Hz, *H*$_3$CCHOH); *N-Acetylglycinamide*, **Ac-Gly-NH₂** (✖) (partial assignment): δ_H 3.90 (2H, s, C*H*$_2$); *N-(2-amino-2-iminoethyl)acetamide*, **Ac-Gly^{N}-NH₂** (✱): δ_H 4.22 (2H, s, C*H*$_2$), 2.10 (3H, s, *H*$_3$C(CO)).

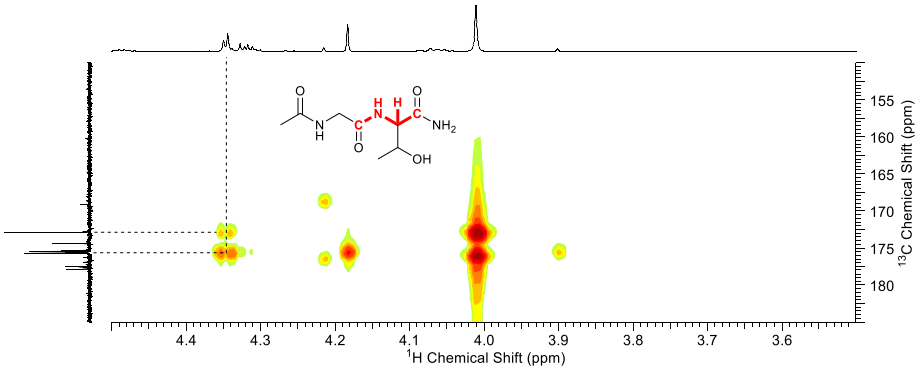


Fig. S169. ^{1}H–^{13}C HMBC (^{1}H: 600 MHz [3.5–4.5 ppm], ^{13}C: 151 MHz [150–185 ppm], H_2O/D_2O 9:1) spectrum showing the diagnostic $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling of the **Thr**-αH-$CONH_2$ in **Ac-Gly-Thr-NH_2** at 4.35 ppm with two resonances at 176 and 173 ppm, which is characteristic of amide bond formation of **Thr-NH_2**. See Fig. S168 for expanded and labelled ^{1}H NMR spectrum.

Coupling of N-acetylglycine nitrile ***Ac-Gly-CN*** *with L-tryptophanamide* ***Trp-NH₂*** *at pH 7 and 60 ℃*

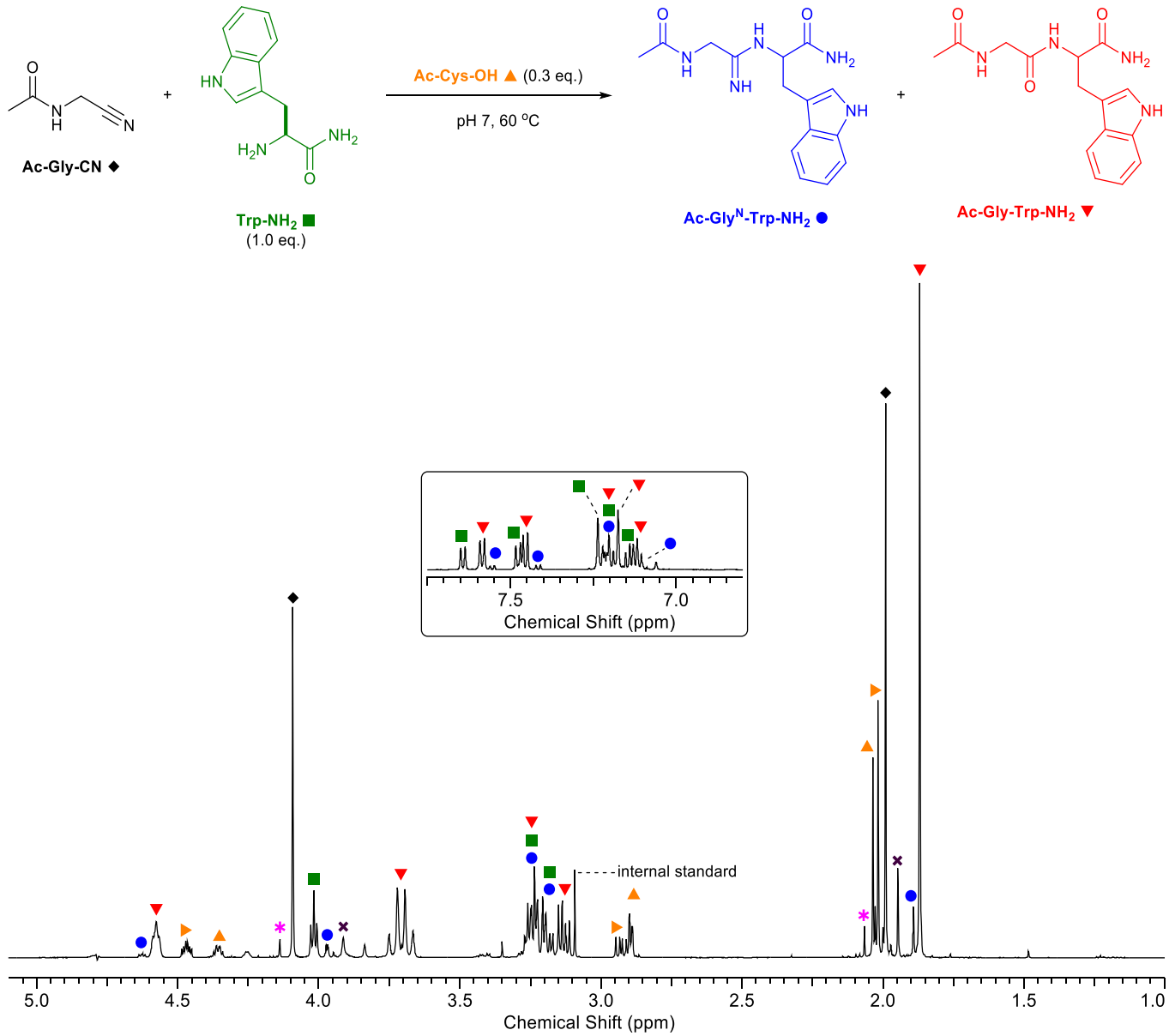


Fig. S170 ^{1}H NMR (600 MHz, H_2O/D_2O 9:1, noesygppr1d, 1.0-5.0 ppm) spectrum to show the reaction of *N*-acetyl glycine nitrile (**Ac-Gly-CN**, 200 mM) with L-tryptophanamide (**Trp-NH₂**, 200 mM) and *N*-acetyl-L-cysteine (**Ac-Cys-OH**, 60 mM, 0.3 eq.) with MSM (5 mM) as the internal standard after 24 h at pH 7 and 60 °C. Inset: ^{1}H NMR (600 MHz, H_2O/D_2O 9:1, noesygppr1d, 6.8-7.75 ppm) showing the aromatic CH resonances present in **Trp-NH₂**, **Ac-Gly^{N}-Trp-NH₂**, and **Ac-Gly-Trp-NH₂**. ▶ = *N,N'*-diacetyl-L-cystine (formed by aerial oxidation of **Ac-Cys-OH**), ✖ = **Ac-Gly-NH₂** and ✱ = **Ac-Gly^{N}-NH₂**.

^{1}H NMR (600 MHz, H_2O/D_2O 9:1, noesygppr1d) *(2-acetamido-1-iminoethyl)tryptophanamide*, **Ac-Gly^{N}-Trp-NH₂** (●) (partial assignment): δ_H 7.55 (1H, d, *J* = 7.8 Hz, Ar*H*), 7.42 (1H, d, *J* = 8.3 Hz, Ar*H*), 7.22-7.19 (2H, m, Ar*H*), 7.16-7.09 (1H, obs., Ar*H*), 4.63 (1H, ABX, *J* = 7.6, 5.2 Hz, Trp-αH-$CONH_2$), 3.97 (2H, app. d, *J* = 3.4 Hz, AcNHCH_2), 1.89 (3H, s, H_3C(CO)); *N-Acetylglycyltryptophanamide*, **Ac-Gly-Trp-NH₂** (▼): δ_H 7.58 (1H, d, *J* = 8.0 Hz, Ar*H*), 7.45 (1H, d, *J* = 8.3 Hz, Ar*H*), 7.22-7.19 (1H, m, Ar*H*), 7.18 (1H, s, Ar*H*), 7.13-7.11 (1H, m, Ar*H*), 4.58 (1H, app. t, *J* = 6.3 Hz, Trp-αH-$CONH_2$), 3.74 (1H, AB, *J* = 17.2 Hz, AcNHCH*H*), 3.68 (1H, AB, *J* = 17.2 Hz, AcNHCH*H*), 3.27-3.22 (1H, m, CHCH*H*Ar), 3.13 (1H, dd, *J* = 14.4, 7.7 Hz, CHCH*H*Ar), 1.87 (3H, s, H_3C(CO)); *L-tryptophanamide*, **Trp-NH₂** (■): δ_H 7.64 (1H, d, *J* = 8.0 Hz, Ar*H*), 7.48 (1H, d, *J* = 8.3 Hz, Ar*H*), 7.24 (1H, s, Ar*H*), 7.22-7.19 (1H, m, Ar*H*), 7.15 (1H, d, *J* = 7.7 Hz, Ar*H*), 4.02 (1H, app. t, *J* = 6.7 Hz, αH-$CONH_2$), 3.27-3.22 (1H, m, CHCH*H*Ar), 3.19 (1H, dd, *J* = 15.3, 6.5 Hz, CHCH*H*Ar); *N-Acetylglycinamide*, **Ac-Gly-NH₂** (✖): δ_H 3.91 (2H, s, CH_2), 1.95 (3H, s, H_3C(CO)); *N-(2-amino-2-iminoethyl)acetamide*, **Ac-Gly^{N}-NH₂** (✱): δ_H 4.14 (2H, s, CH_2), 2.06 (3H, s, H_3C(CO)).

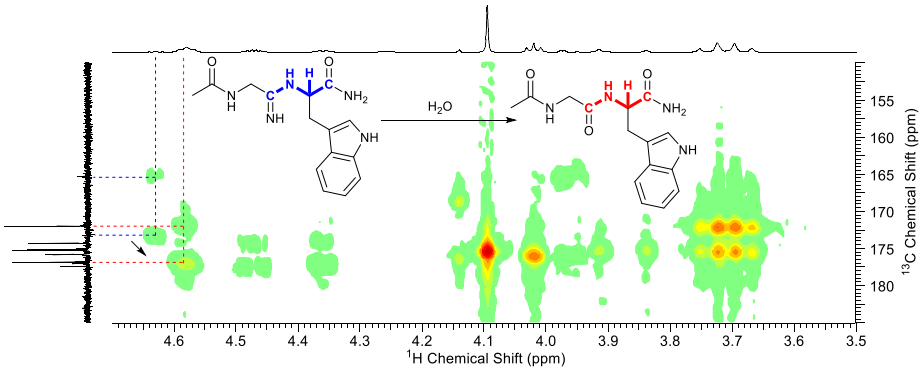


Fig. S171. ^{1}H–^{13}C HMBC (^{1}H: 600 MHz [3.5–4.7 ppm], ^{13}C: 151 MHz [150–185 ppm], H_2O/D_2O 9:1) spectrum showing the diagnostic $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling of the **Trp**-αH-$CONH_2$ in **Ac-Gly^{N}-Trp-NH$_2$** at 4.63 ppm with two resonances at 173 and 165 ppm, which is characteristic of amidine bond formation of **Trp-NH$_2$**, and the diagnostic $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling of the **Trp**-αH-$CONH_2$ in **Ac-Gly-Trp-NH$_2$** at 4.58 ppm with two resonances at 177 and 172 ppm, which is characteristic of amide bond formation of **Trp-NH$_2$**. See Fig. S170 for expanded and labelled ^{1}H NMR spectrum.

Coupling of N-acetylglycine nitrile ***Ac-Gly-CN*** *with L-tyrosinamide* ***Tyr-NH₂*** *at pH 7 and 60*

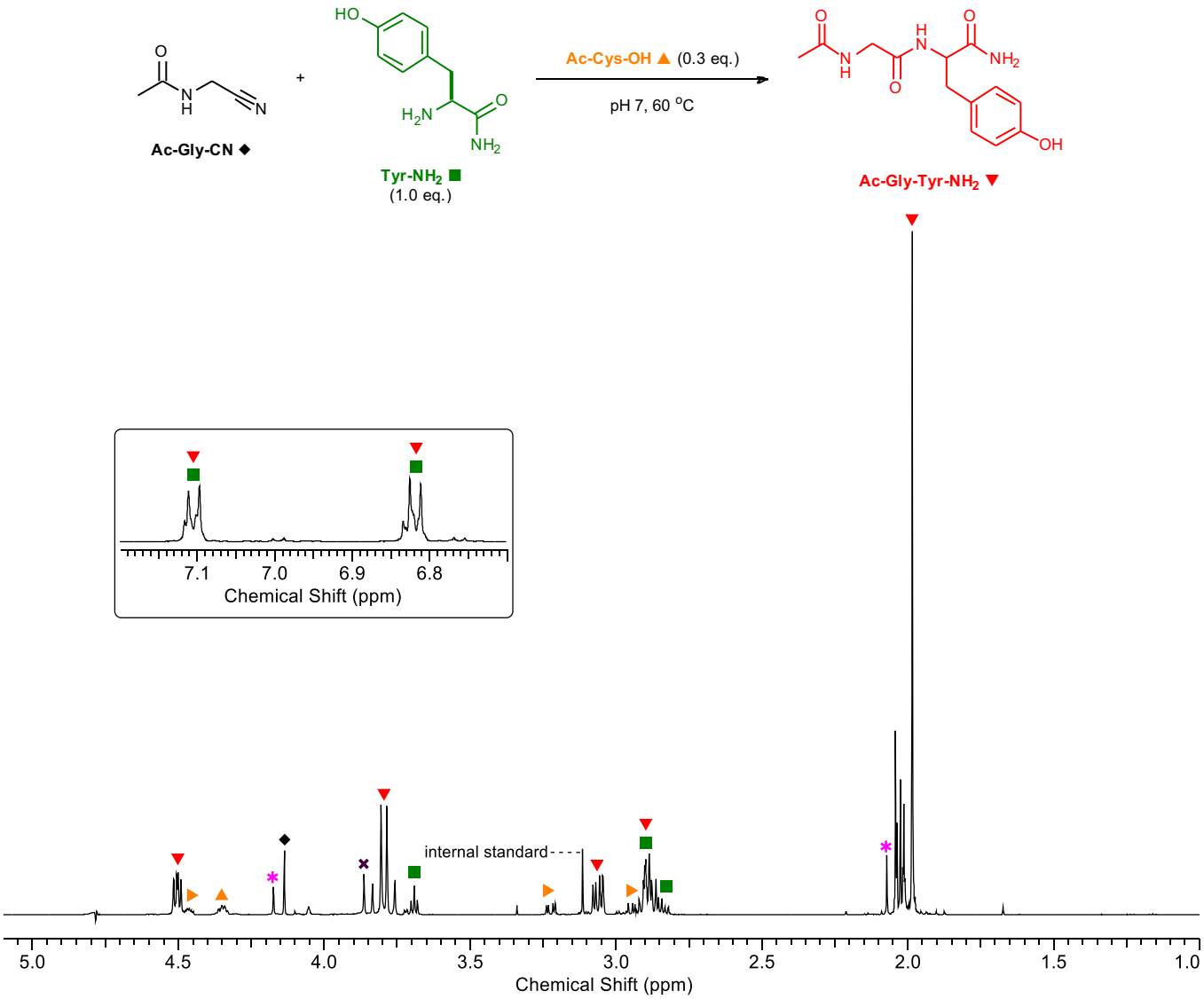


Fig. S172. ^{1}H NMR (600 MHz, H_2O/D_2O 9:1, noesygppr1d, 1.0–5.0 ppm) spectrum to show the reaction of *N*-acetyl glycine nitrile (**Ac-Gly-CN**, 200 mM) with L-tyrosinamide (**Tyr-NH₂**, 200 mM) and *N*-acetyl-L-cysteine (**Ac-Cys-OH**, 60 mM, 0.3 eq.) with MSM (5 mM) as the internal standard after 24 h at pH 7 and 60 °C. ▶ = *N,N′*-diacetyl-L-cystine (formed by aerial oxidation of **Ac-Cys-OH**), ✖= **Ac-Gly-NH₂** and ✱= **Ac-Gly^{N}-NH₂.**

^{1}H NMR (600 MHz, H_2O/D_2O 9:1, noesygppr1d) *N-Acetylglycyltyrosinamide*, **Ac-Gly-Tyr-NH₂** (▼): δ_H 7.10 (2H, d, *J* = 8.4 Hz, Ar*H*), 6.82 (2H, d, *J* = 8.5 Hz, Ar*H*), 4.50 (1H,ABX, *J* = 8.1, 5.9 Hz, Tyr-αH-CONH₂), 3.82 (1H, AB, *J* = 16.9 Hz, AcNHCH*H*), 3.77 (1H, AB, *J* = 16.9 Hz, AcNHCH*H*), 3.06 (1H, ABX, *J* = 13.9, 5.9 Hz, CHCH*H*Ar), 2.88 (1H, ABX, *J* = 13.9, 8.1 Hz, CHCH*H*Ar), 1.98 (3H, s, H_3C(CO)); *L-tyrosinamide*, **Tyr-NH₂** (■): δ_H 7.91 (2H, d, *J* = 9.1 Hz, Ar*H*), 6.83 (2H, m, Ar*H*), 3.69 (1H, t, *J* = 6.8 Hz, αH-CONH₂), 2.90 (1H, m, CHCH*H*Ar), 2.84 (1H, dd, *J* = 13.9, 6.8 Hz, CHCH*H*Ar); *N-Acetylglycinamide*, **Ac-Gly-NH₂** (✖) (partial assignment): δ_H 3.86 (2H, s, CH_2); *N-(2-amino-2-iminoethyl)acetamide*, **Ac-Gly^{N}-NH₂** (✱): δ_H 4.17 (2H, s, CH_2), 2.07 (3H, s, H_3C(CO)).

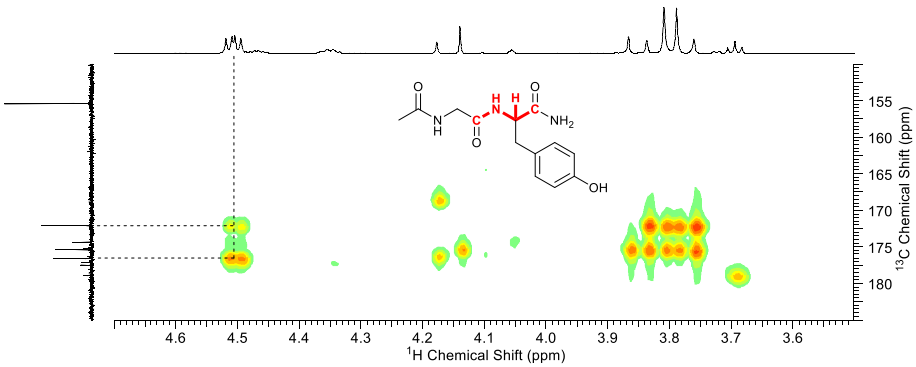


Fig. S173. ^{1}H–^{13}C HMBC (^{1}H: 600 MHz [3.5–4.7 ppm], ^{13}C: 151 MHz [150–185 ppm], H_2O/D_2O 9:1) spectrum showing the diagnostic $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling of the **Tyr**-αH-$CONH_2$ in **Ac-Gly-Tyr-NH_2** at 4.50 ppm with two resonances at 177 and 172 ppm, which is characteristic of amide bond formation of **Tyr-NH_2**. See Fig. S172 for expanded and labelled ^{1}H NMR spectrum.

Coupling of N-acetylglycine nitrile ***Ac-Gly-CN*** *with D-valinamide D-****Val-NH₂*** *at pH 7 and 60 °C*

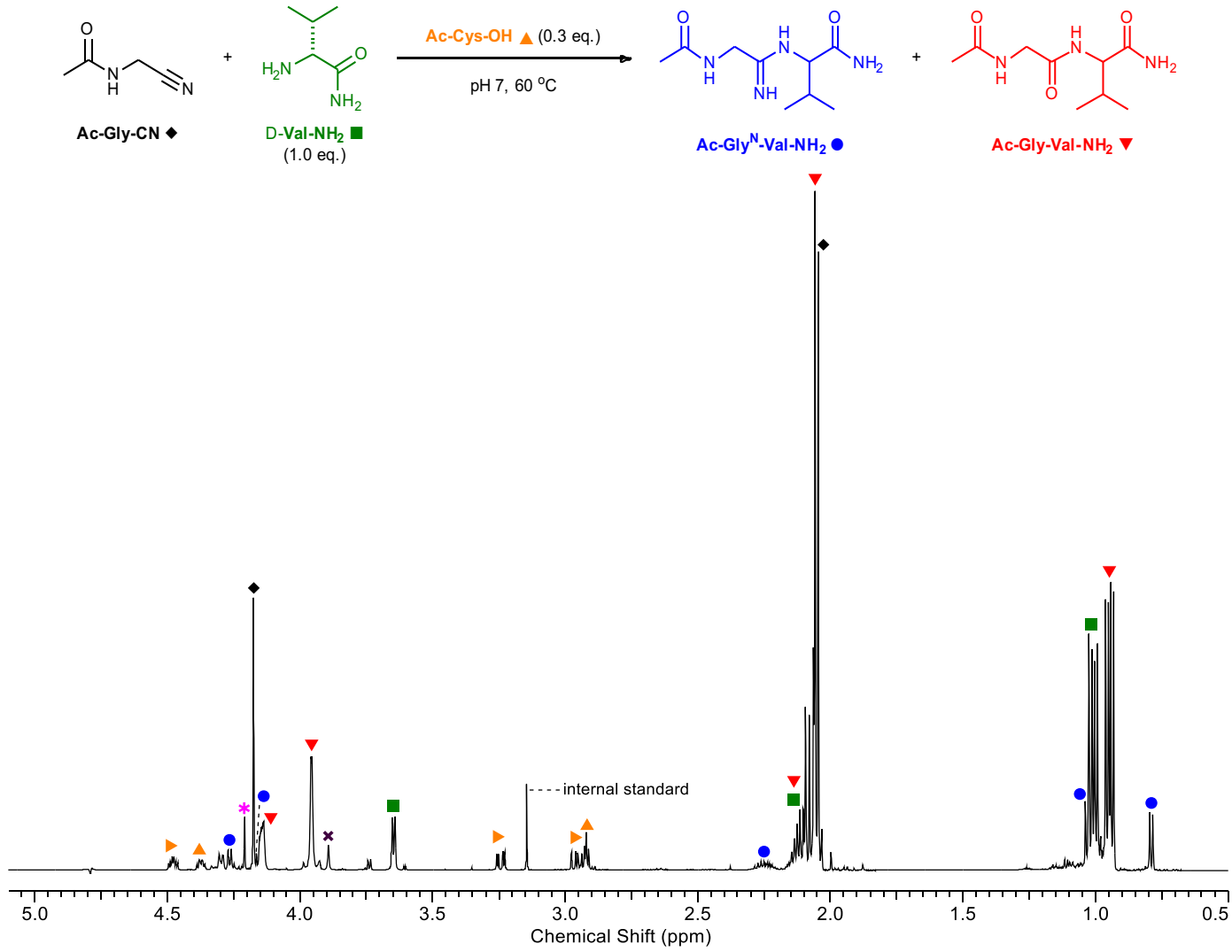


Fig. S174. ^{1}H NMR (600 MHz, H_2O/D_2O 9:1, noesygppr1d, 0.5-5.0 ppm) spectrum to show the reaction of *N*-acetyl glycine nitrile (**Ac-Gly-CN**, 200 mM) with D-valinamide (D-**Val-NH₂**, 200 mM) and *N*-acetyl-L-cysteine (**Ac-Cys-OH**, 60 mM, 0.3 eq.) with MSM (5 mM) as the internal standard after 24 h at pH 7 and 60 °C. ▶ = *N,N′*-diacetyl-L-cystine (formed by aerial oxidation of **Ac-Cys-OH**), ✖= **Ac-Gly-NH₂** and ✱= **Ac-Gly^N-NH₂.**

^{1}H NMR (600 MHz, H_2O/D_2O 9:1, noesygppr1d) *(2-acetamido-1-iminoethyl)valinamide*, **Ac-Gly^N-Val-NH₂** (●) (partial assignment): δ_H 4.27 (2H, app. d, *J* = 6.6 Hz, AcNHC*H*$_2$), 4.16 (1H, m, Val-αH-CONH$_2$), 2.30-2.20 (1H, m, H_3CC*H*CH$_3$), 1.03 (3H, obs., *H*$_3$CCHCH$_3$), 0.79 (3H, d, *J* = 6.9 Hz, *H*$_3$CCHCH$_3$); *N-Acetylglycylvalinamide*, **Ac-Gly-Val-NH₂** (▼) (partial assignment): δ_H 4.15-4.14 (1H, m, Val-αH-CONH$_2$), 3.97 (1H, AB, *J* = 17.3 Hz, AcNHCH*H*), 3.94 (1H, AB, *J* = 17.3 Hz, AcNHCH*H*), 2.15-2.07 (1H, m, H_3CC*H*CH$_3$), 0.96 (3H, d, *J* = 6.9 Hz, *H*$_3$CCHCH$_3$), 0.94 (3H, d, *J* = 6.9 Hz, *H*$_3$CCHCH$_3$); *D-valinamide*, D-**Val-NH₂** (■): δ_H 3.65 (1H, d, *J* = 5.8 Hz, αH-CONH$_2$), 2.15-2.07 (1H, m, H_3CC*H*CH$_3$), 1.02 (3H, d, *J* = 7.0 Hz, *H*$_3$CCHCH$_3$), 1.00 (3H, d, *J* = 7.0 Hz, *H*$_3$CCHCH$_3$); *N-Acetylglycinamide*, **Ac-Gly-NH₂** (✖) (partial assignment): δ_H 3.89 (2H, s, C*H*$_2$); *N-(2-amino-2-iminoethyl)acetamide*, **Ac-Gly^N-NH₂** (✱): δ_H 4.21 (2H, s, C*H*$_2$).

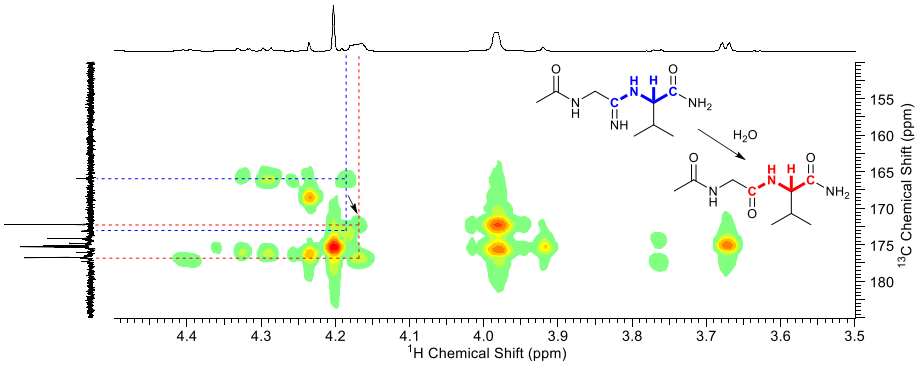
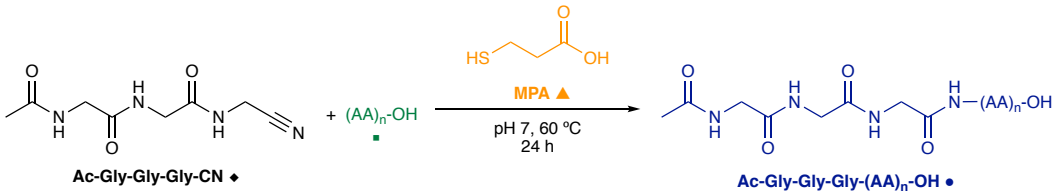


Fig. S175. ^{1}H–^{13}C HMBC (^{1}H: 600 MHz [3.5–4.5 ppm], ^{13}C: 151 MHz [150–185 ppm], H_2O/D_2O 9:1) spectrum showing the diagnostic $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling of the **Val**-αH-$CONH_2$ in **Ac-Gly^{N}-Val-NH$_2$** at 4.16 ppm with two resonances at 173 and 166 ppm, which is characteristic of amidine bond formation of D-**Val-NH$_2$**, and the diagnostic $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling of the **Val**-αH-$CONH_2$ in **Ac-Gly-Val-NH$_2$** at 4.15-4.14 ppm with two resonances at 177 and 172 ppm, which is characteristic of amide bond formation of D-**Val-NH$_2$**. See Fig. S174 for expanded and labelled ^{1}H NMR spectrum.

Prebiotic thiol-catalysed peptide fragment ligations



N-Acetylglycylglycylglycine nitrile **Ac-Gly$_3$-CN** (*4*) (100 mM) and peptide **AA$_n$-OH** (100 mM) and 3-mercaptopropionic acid (160 mM) were dissolved in H_2O and the solution was adjusted to pH 7.0 with 4 M NaOH**.** The resulting solution was then heated at 60 °C for 24 h. The reaction was cooled to room temperature and analysed by NMR spectroscopy (^{1}H; ^{13}C; ^{1}H–^{1}H COSY; ^{1}H–^{13}C HSQC; ^{1}H–^{13}C HMBC). The presence of ligation product **Ac-Gly$_3$-AA$_n$-OH** was quantified using relative integral analysis by ^{1}H NMR, ^{1}H–^{13}C HMBC NMR correlation analysis, and high-resolution mass spectrometry. Yields and HRMS-ESI data are given in Table S8. *N*-Acetylpeptides were then precipitated by dilution of the reaction mixtures with acetone (**Ac-Gly-Gly-Gly-Ala-Ala-Ala-OH** and **Ac-Gly-Gly-Gly-Gly-Ala-Gly-OH**) or ethanol (**Ac-Gly-Gly-Gly-Gly-Gly-Gly-OH**) until precipitate was observed. The precipitate was isolated by centrifugation and then dried *in vacuo* to give a white powder.

Entry	(AA)$_n$-OH	Ac-(Gly)$_n$-(AA)$_n$-OH (%)	HRMS-ESI Data for Ac-(Gly)$_n$-(AA)$_n$-X		
			Formula	Calculated	Found
1	Met-Gly-OH	80	$C_{15}H_{26}N_5O_7S$ $[M+H]^+$	420.1547	420.1552
2	Ala-Ala-Ala-OH	90	$C_{17}H_{29}N_6O_8$ $[M+H]^+$	445.2041	445.2044
3	Ala-Gly-Ala-OH	84	$C_{16}H_{27}N_6O_8$ $[M+H]^+$	431.1885	431.1893
4	Gly-Ala-Gly-OH	87	$C_{15}H_{25}N_6O_8$ $[M+H]^+$	417.1728	417.1739
5	Gly-Gly-Gly-OH	89	$C_{14}H_{23}N_6O_8$ $[M+H]^+$	403.1572	403.1570
6	Gly-Gly-His-OH	89	$C_{18}H_{27}N_8O_8$ $[M+H]^+$	483.1946	483.1968
7	Leu-Leu-Leu-OH[a]	76	$C_{26}H_{47}N_6O_8$ $[M+H]^+$	571.3450	571.3460
8	Met-Ala-Ser-OH	77	$C_{19}H_{33}N_6O_9S$ $[M+H]^+$	521.2024	521.2022
9	Phe-Gly-Gly-OH	77[b]	$C_{21}H_{27}N_6O_8$ $[M-H]^-$	491.1889	491.1890

Table S8. NMR yields and high resolution mass spectrometry (electrospray ionization) data of 3-mercaptopropionic acid (**MPA**; 160 mM) catalysed coupling of **Ac-Gly$_3$-CN** (100 mM) with peptide **AA$_n$-OH** (100 mM) after heating at 60 °C for 24 h at pH 7.0 in water. [a] Reaction with **Leu-Leu-Leu-OH** heated at 60 °C for 48 h at pH 7.0 in water. [b] **Ac-Gly-Gly-Gly^N-Phe-Gly-Gly-OH** (12%) was also observed. Total ligation yield was 89%.

N-Acetylglycylglycylglycyl-DL-methioninylglycine ***Ac-Gly-Gly-Gly-Met-Gly-OH***

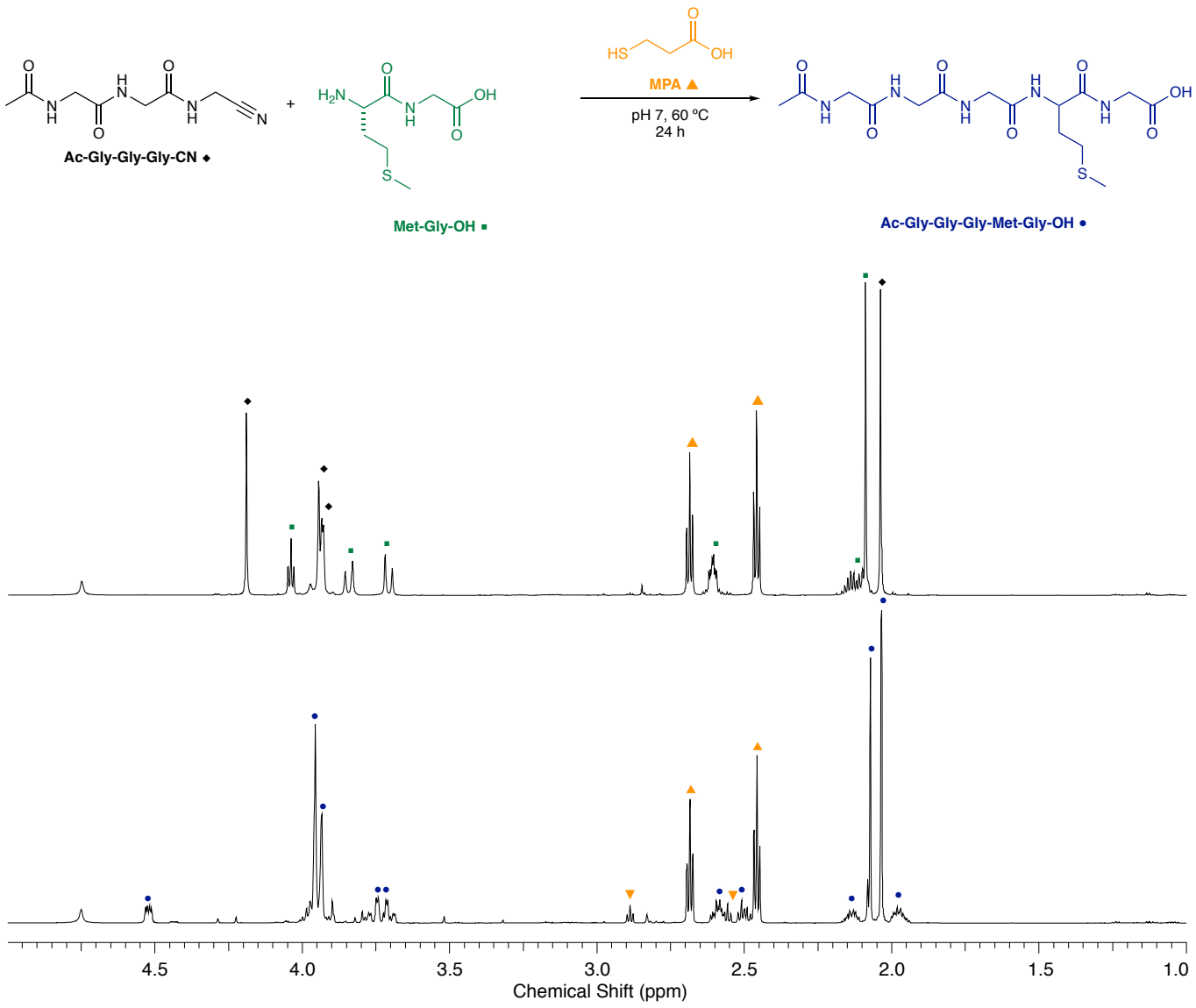


Fig. S176. ^{1}H NMR (700 MHz, H_2O, noesygppr1d, 1.0–5.0 ppm) spectrum to show the reaction of *N*-acetylglycylglycylglycine nitrile(**Ac-Gly$_3$-CN**; 100 mM), L-methionylglycine (**Met-Gly-OH**; 100 mM) and 3-mercaptopropionic acid (**MPA**; 160 mM) in H_2O (1 mL) at pH 7 at 60 °C. ▼ = 2-Carboxyethyldisulfide (formed by aerial oxidation of **MPA** ▲). Top = ^{1}H NMR spectrum acquisition before the reaction was heated 60 °C. Bottom = ^{1}H NMR spectrum acquisition after the reaction was heated 60 °C for 24 h.

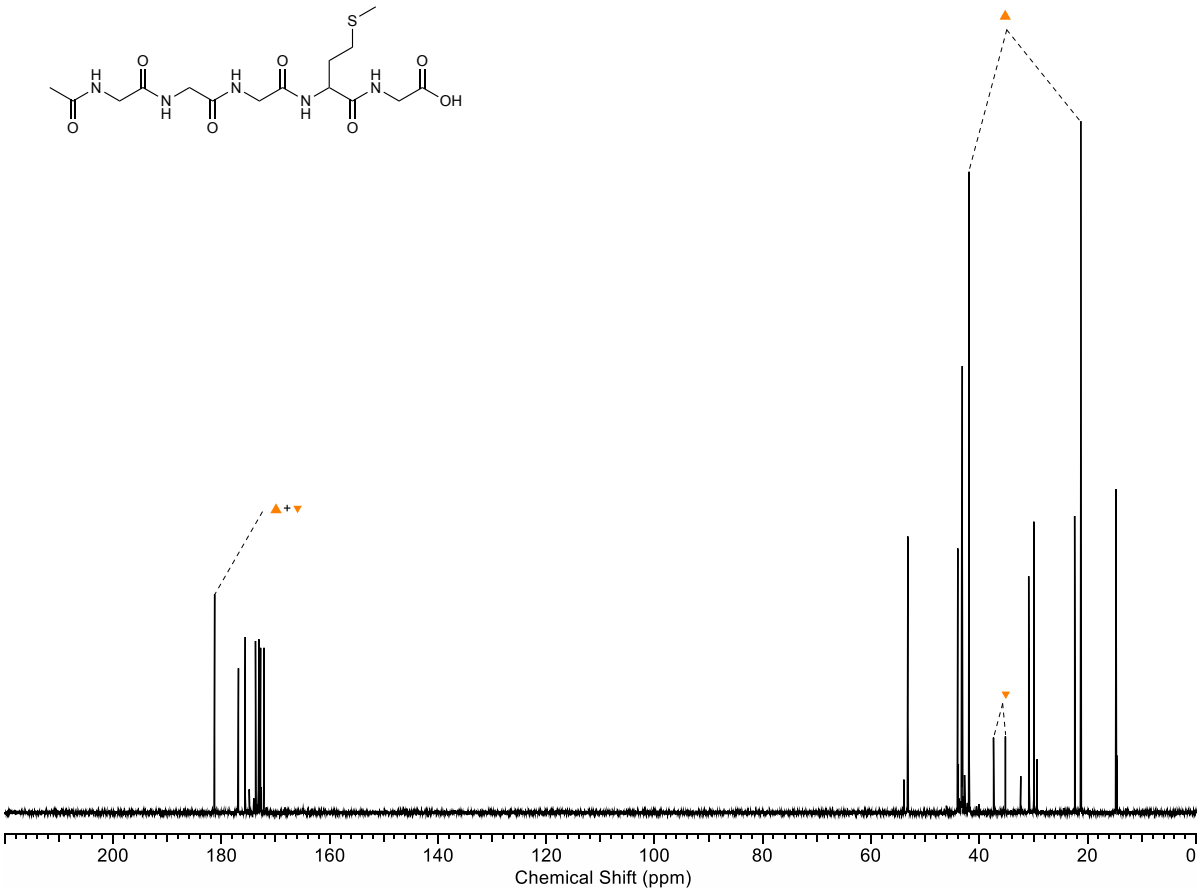


Fig. S177. ^{13}C NMR (176 MHz, H_2O, 0–220 ppm) spectrum to show the reaction of *N*-acetylglycylglycylglycine nitrile(**Ac-Gly$_3$-CN**; 100 mM), methionylglycine (**Met-Gly-OH**; 100 mM) and 3-mercaptopropionic acid (**MPA**; 160 mM) in H_2O (1 mL) at pH 7 at 60 °C. ▼ = 2-Carboxyethyldisulfide (formed by aerial oxidation of **MPA** ▲).

^{1}H NMR (700 MHz, H_2O, noesygppr1d): *N-Acetylglycylglycylglycyl-DL-methionylglycine* ***Ac-Gly-Gly-Gly-Met-Gly-OH*** (•) (Fig. S176): δ 4.52 (dd, *J* = 4.5, 9.6 Hz, 1H, Met-(C2)−H), 3.96 (s, 4H, Gly$_2$-(C2)−H$_2$, Gly$_3$-(C2)−H$_2$), 3.93 (s., 2H, Gly$_1$-(C2)−H$_2$), 3.78 - 3.73 (m, 1H, Gly$_5$-(C2)−H$_a$), 3.73 - 3.68 (m, 1H, Gly$_5$-(C2)−H$_b$), 2.63 - 2.57 (m, 1H, Met-(C4)−H$_a$), 2.53 - 2.47 (m, 1H, Met-(C4)−H$_b$), 2.18 - 2.10 (m, 1H, Met-(C3)−H$_a$), 2.07 (s, 3H, -SCH$_3$), 2.04 (s, 3H, COCH$_3$), 2.01 - 1.93 (m, 1H, Met-(C3)−H$_b$). ^{13}C NMR (176 MHz, H_2O, noesygppr1d) (Fig. S177): δ 176.8 (Gly$_5$-(C1)), 175.6 (**C**OCH$_3$), 173.7 (CO), 173.1 (CO), 172.8 (CO), 172.2 (CO), 53.2 (Met-(C2)), 44.0 (Gly$_5$-(C2)), 43.3 (Gly-(C2)), 43.2 (Gly-(C2) × 2)), 30.9 (Met-(C3)), 29.9 (Met-(C4)), 22.4 (CO**C**H$_3$), 14.7 (SCH$_3$).

N-Acetylglycylglycylglycyl-DL-alanyl-L-alanyl-L-alanine ***Ac-Gly-Gly-Gly-Ala-Ala-Ala-OH***

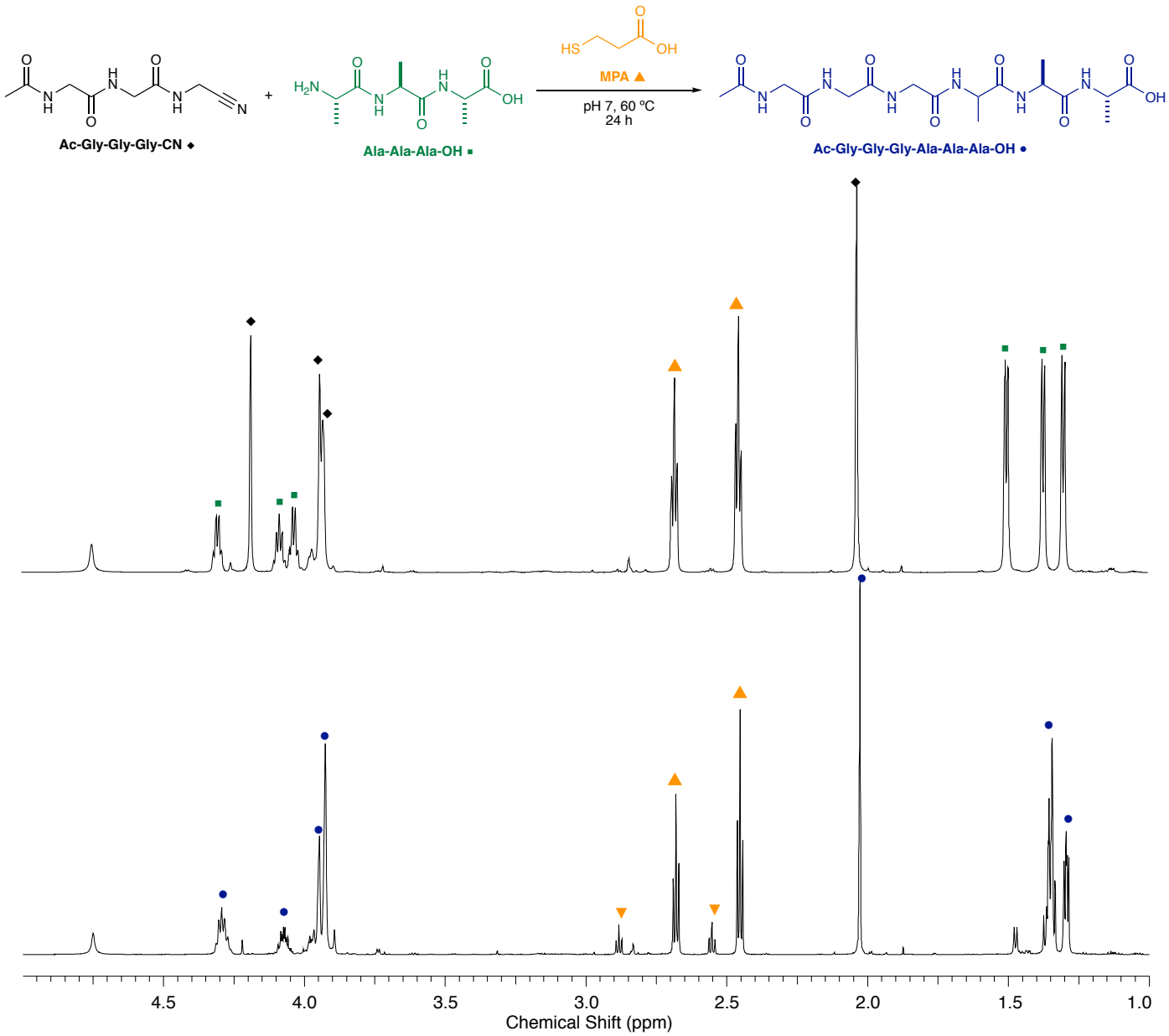


Fig. S178. ^{1}H NMR (700 MHz, H_2O, noesygppr1d, 1.0–5.0 ppm) spectrum to show the reaction of *N*-acetylglycylglycylglycine nitrile (**Ac-Gly$_3$-CN**; 100 mM), L-alanyl-L-alanyl-L-alanine (**Ala-Ala-Ala-OH**; 100 mM) and 3-mercaptopropionic acid (**MPA**; 160 mM) in H_2O (1 mL) at pH 7 at 60 °C. ▼ = 2-Carboxyethyldisulfide (formed by aerial oxidation of **MPA** ▲). Top = ^{1}H NMR spectrum acquisition before the reaction was heated 60 °C. Bottom = ^{1}H NMR spectrum acquisition after the reaction was heated 60 °C for 24 h.

^{1}H NMR (700 MHz, H_2O, noesygppr1d): *N*-Acetylglycylglycylglycyl-DL-alanyl-L-alanyl-L-alanine ***Ac-Gly-Gly-Gly-Ala-Ala-Ala-OH*** (●) (2 diastereoisomers) (Fig. S178): δ 4.33 - 4.25 (m, 4H, 2 × Ala-(C2)−H), 4.11 - 4.03 (m, 2H, Ala-(C2)−H), 3.95 (br. s., 4H, Gly-(C2)–H_2), 3.93 (br. s., 8H, 2 × Gly-(C2)–H_2), 2.03 (s, 6H, $COCH_3$), 1.39 - 1.32 (m, 12H, 4 × Ala-(C3)–H_3), 1.31 - 1.27 (m, 6H, 2 × Ala-(C3)–H_3).

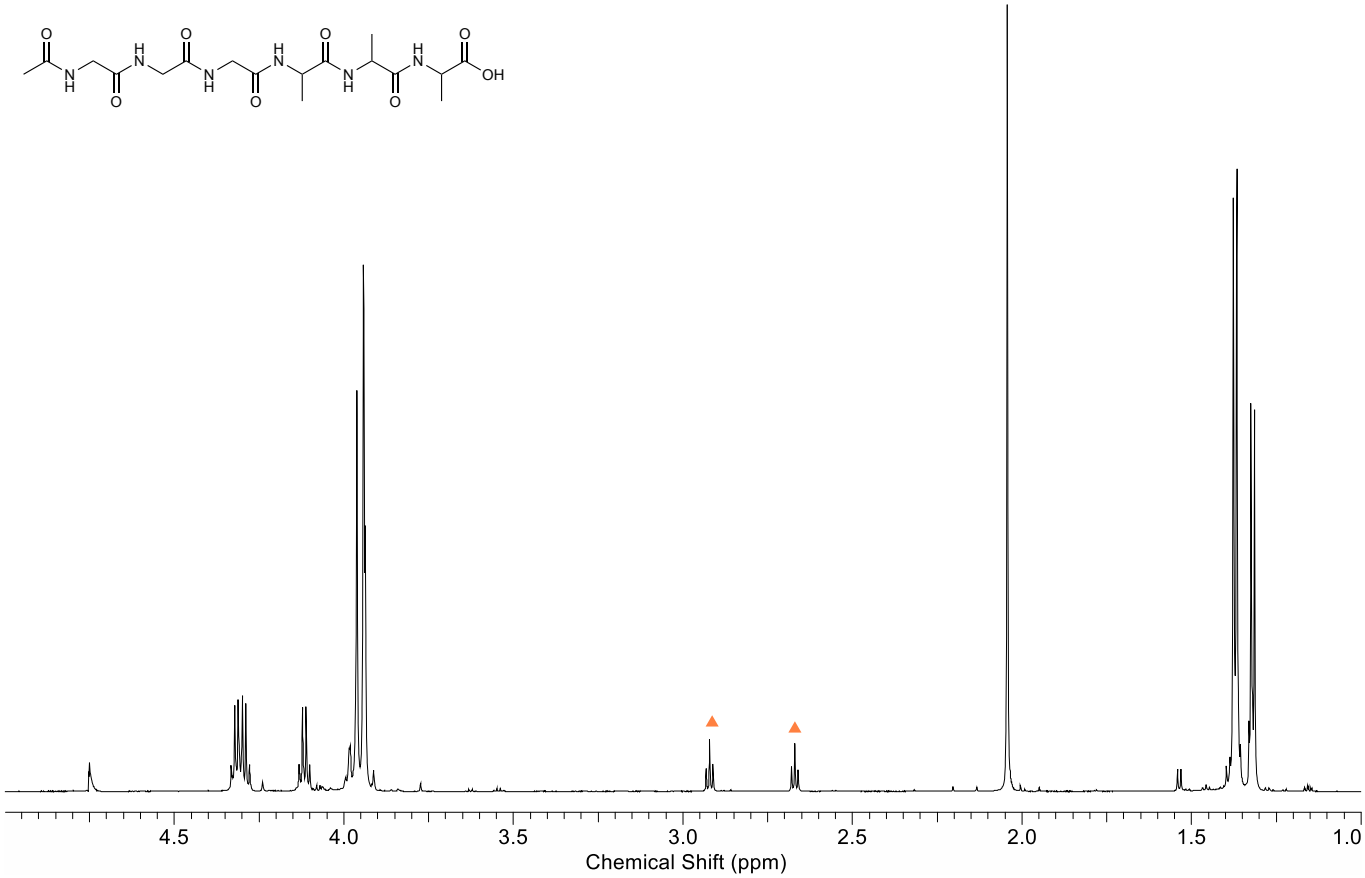


Fig. S179. ^{1}H NMR (700 MHz, D_2O, noesygppr1d, 1.0–5.0 ppm) spectrum to show a single diastereoisomer of **Ac-Gly-Gly-Gly-Ala-Ala-Ala-OH** isolated after precipitation by acetone from the crude mixture obtained from the reaction of *N*-acetylglycylglycylglycine nitrile (**Ac-Gly$_3$-CN**; 100 mM), L-alanyl-L-alanyl-L-alanine (**Ala-Ala-Ala-OH**; 100 mM) and 3-mercaptopropionic acid (**MPA**; 160 mM) in H_2O (1 mL) at pH 7 at 60 °C. ▼ = residual 2-carboxyethyldisulfide (formed by aerial oxidation of **MPA** ▲).

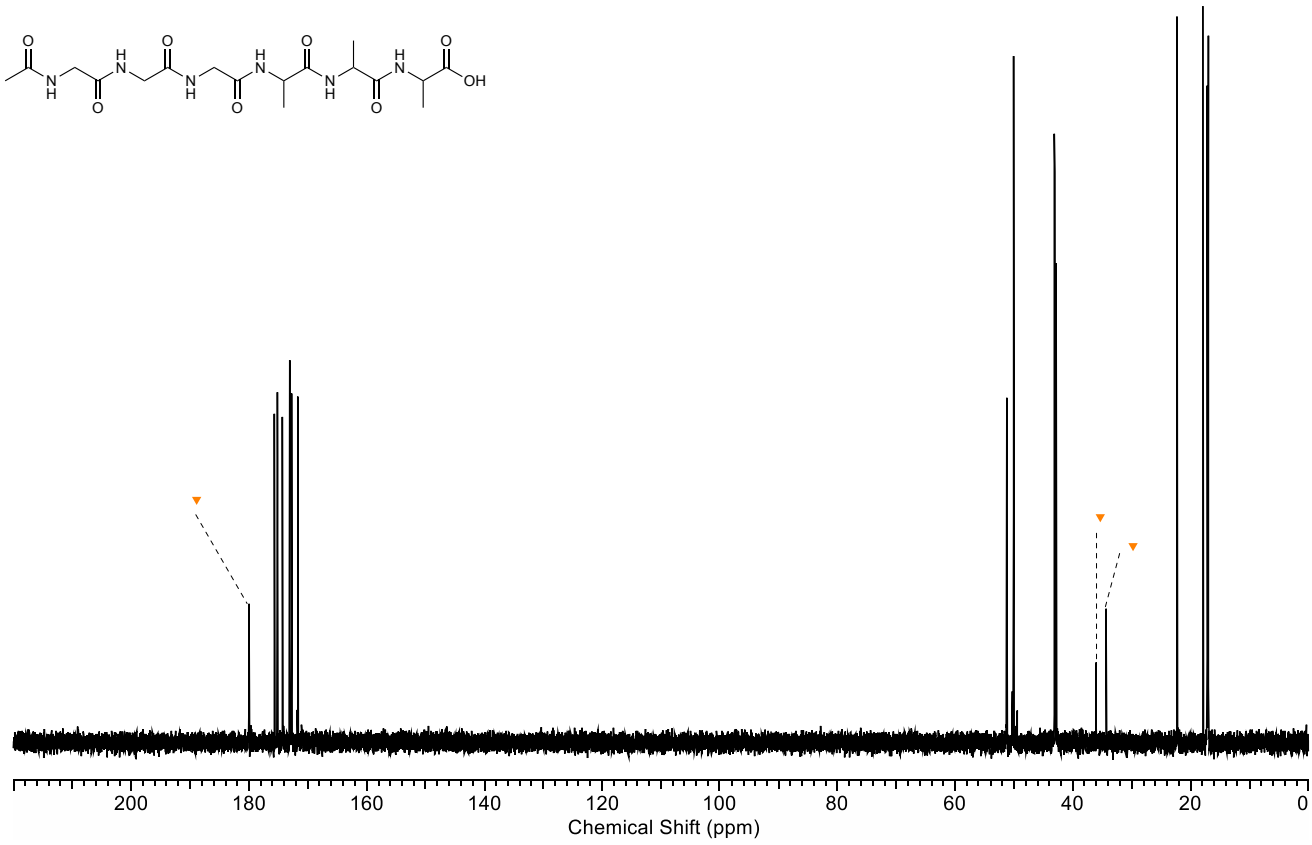


Fig. S180. ^{13}C NMR 176 MHz, D_2O, 0–220 ppm) spectrum to show a single diastereoisomer of **Ac-Gly-Gly-Gly-Ala-Ala-Ala-OH** isolated after precipitation by acetone from the crude mixture obtained from the reaction of *N*-acetylglycylglycylglycine nitrile (**Ac-Gly$_3$-CN**; 100 mM), L-alanyl-L-alanyl-L-alanine (**Ala-Ala-Ala-OH**; 100 mM) and 3-mercaptopropionic acid (160 mM) in H_2O (1 mL) at pH 7 at 60 °C. ▼ = residual 2-Carboxyethyldisulfide (formed by aerial oxidation of **MPA** ▲).

N-Acetylglycylglycylglycylalanylalanylalanine ***Ac-Gly-Gly-Gly-Ala-Ala-Ala-OH*** (single diastereoisomer) (Fig. S179): ^{1}H NMR (700 MHz, D_2O) δ 4.34 - 4.27 (m, 2H, 2 $\times$ Ala-(C2)–H), 4.12 (q, J = 7.2 Hz, 1H, Ala-(C2)–H), 3.96 (s, 2H, Gly-(C2)–H_2), 3.95 - 3.93 (2 $\times$ s , 4H, 2 $\times$ Gly-(C2)–H_2), 2.04 (s, 3H, $COCH_3$), 1.37 (d, J = 7.4 Hz, 6H, 2 $\times$ Ala-(C3)–H_3), 1.33 - 1.31 (d, J = 7.4 Hz, 3H, Ala-(C3)–H_3). ^{13}C NMR (176 MHz, D_2O) (Fig. S180): δ 180.0 (Ala-(C1)), 175.6 (**C**OCH_3), 175.2 (Ala-(C1)),174.3 (Ala-(C1)), 173.1 (Gly-(C1)), 172.7 (Gly-(C1)), 171.7 (Gly-(C1)), 51.3 (Ala-(C2)), 50.1 (Ala-(C2)), 50.1 (Ala-(C2)), 43.1 (Gly-(C2)), 43.1 (Gly-(C2)), 42.8 (Gly-(C2)), 22.3 (CO**C**H_3), 17.9 (Ala-(C3)), 17.2 (Ala-(C3)), 17.0 (Ala-(C3)).

N-Acetylglycylglycylglycyl-DL-alanylglycyl-L-alanine ***Ac-Gly-Gly-Gly-Ala-Gly-Ala-OH***

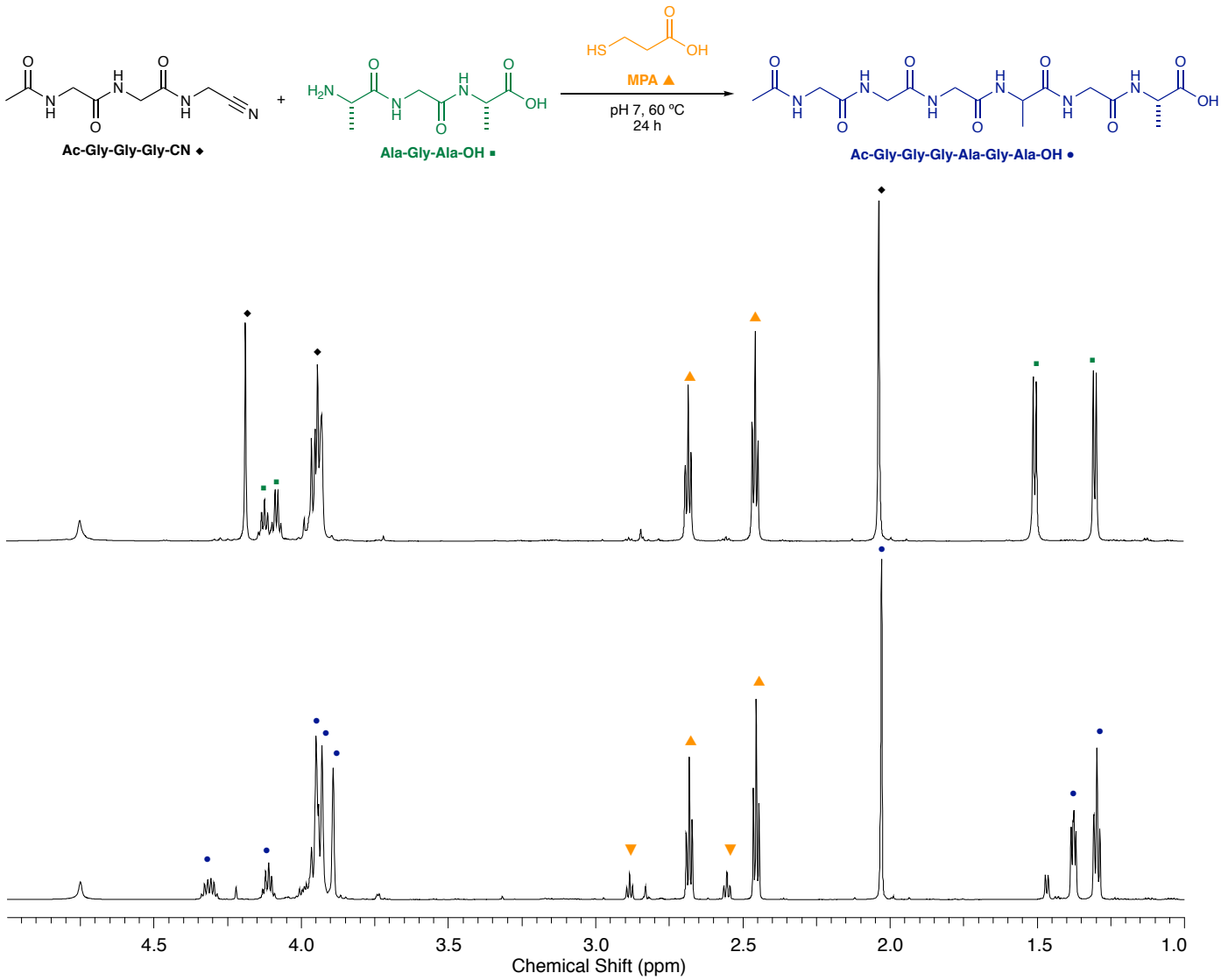


Fig. S181. ^{1}H NMR (700 MHz, H_2O, noesygppr1d, 1.0–5.0 ppm) spectrum to show the reaction of *N*-acetylglycylglycylglycine nitrile(**Ac-Gly$_3$-CN**; 100 mM), L-alanylglycyl-L-alanine (**Ala-Gly-Ala-OH**; 100 mM) and 3-mercaptopropionic acid (**MPA**; 160 mM) in H_2O (1 mL) at pH 7 at 60 °C. ▼ = 2-Carboxyethyldisulfide (formed by aerial oxidation of **MPA** ▲). Top = ^{1}H NMR spectrum acquisition before the reaction was heated 60 °C. Bottom = ^{1}H NMR spectrum acquisition after the reaction was heated 60 °C for 24 h.

N-Acetylglycylglycylglycyl-DL-alanylglycyl-DL-alanine ***Ac-Gly-Gly-Gly-Ala-Gly-Ala-OH*** (2 diastereoisomers) (●) (Fig. S181): ^{1}H NMR (700 MHz, H_2O) δ 4.35 - 4.28 (m, 2H, Ala-(C2)–H), 4.15 - 4.08 (m, 2H, Ala-(C2)–H), 3.98 - 3.93 (m, 8H, 2 × Gly-(C2)–H), 3.93 (s, 4H, Gly-(C2)–H), 3.89 (s, 4H, Gly-(C2)–H), 2.03 (s, 6H, $COCH_3$),1.38 - 1.35 (m, 6H, Ala-(C3)–H_3), 1.31 - 1.29 (m, 6H, Ala-(C3)–H_3).

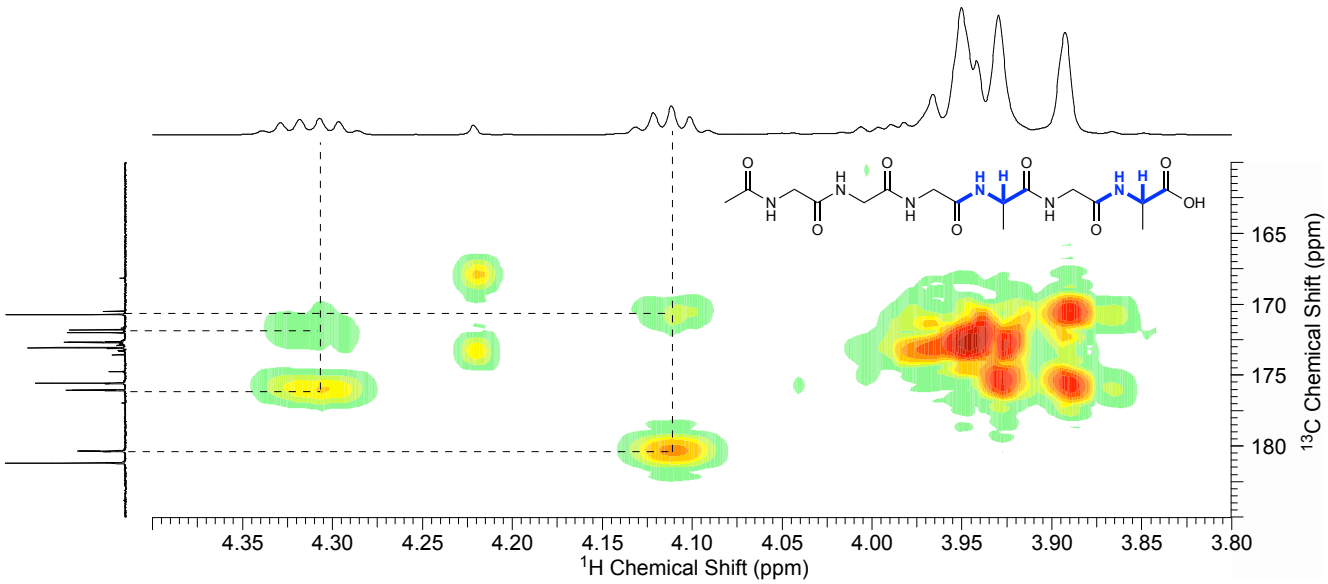


Fig. S182. ^{1}H–^{13}C HMBC (^{1}H: 700 MHz [3.80–4.40 ppm], ^{13}C: 176 MHz [160-185 ppm]) spectrum showing the diagnostic $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling of **Ala$_4$**-(C2)–H and **Ala$_6$**-(C2)–H in a diastereoisomeric mixture of **Ac-Gly-Gly-Gly-Ala-Gly-Ala-OH** (δ_H = 4.31 ppm, δ_c = 176.1, 176.0, 171.8, 171.9; δ_H = 4.11 ppm, δ_c 170.7 (× 2), 180.37, 180.34 ppm.). See Fig. S181 for expanded and labelled ^{1}H NMR spectrum.

N-Acetylglycylglycylglycylglycylglycylglycylglycine ***Ac-Gly-Gly-Gly-Gly-Gly-Gly-OH***

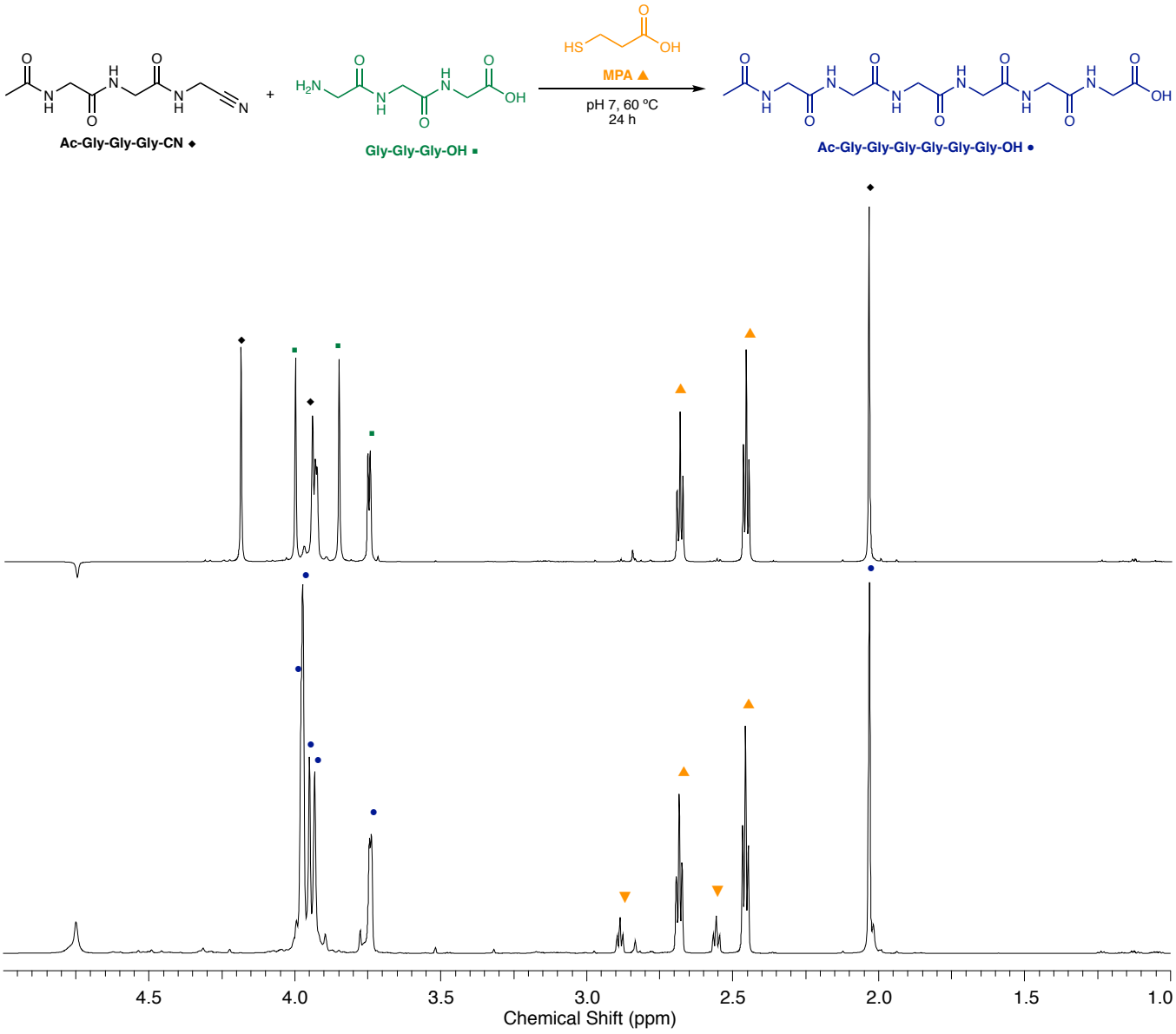


Fig. S183. ^{1}H NMR (700 MHz, H_2O, noesygppr1d, 1.0–5.0 ppm) spectrum to show the reaction of *N*-acetylglycylglycylglycylglycine nitrile(**Ac-Gly$_3$-CN**; 100 mM), glycylglycylglycine (**Gly-Gly-Gly-OH**; 100 mM) and 3-mercaptopropionic acid (**MPA**; 160 mM) in H_2O (1 mL) at pH 7 at 60 °C. ▼ = 2-Carboxyethyldisulfide (formed by aerial oxidation of **MPA** ▲). Top = ^{1}H NMR spectrum acquisition before the reaction was heated 60 °C. Bottom = ^{1}H NMR spectrum acquisition after the reaction was heated 60 °C for 24 h.

^{1}H NMR (700 MHz, H_2O, noesygppr1d): *N*-Acetylglycylglycylglycylglycylglycylglycylglycine ***Ac-Gly-Gly-Gly-Gly-Gly-Gly-OH*** (●) (Fig. S183): δ 3.98 (br. s., 2H, Gly-(C2)–H_2), 3.97 (br. s., 4H, Gly-(C2)–H_2 × 2), 3.95 (s, 2H, Gly-(C2)–H_2), 3.93 (s, 2H, Gly-(C2)–H_2), 3.74 (app. d., *J* = 5.2 Hz, 2H, Gly-(C2)–H_2), 2.03 (s, 3H, $COCH_3$)

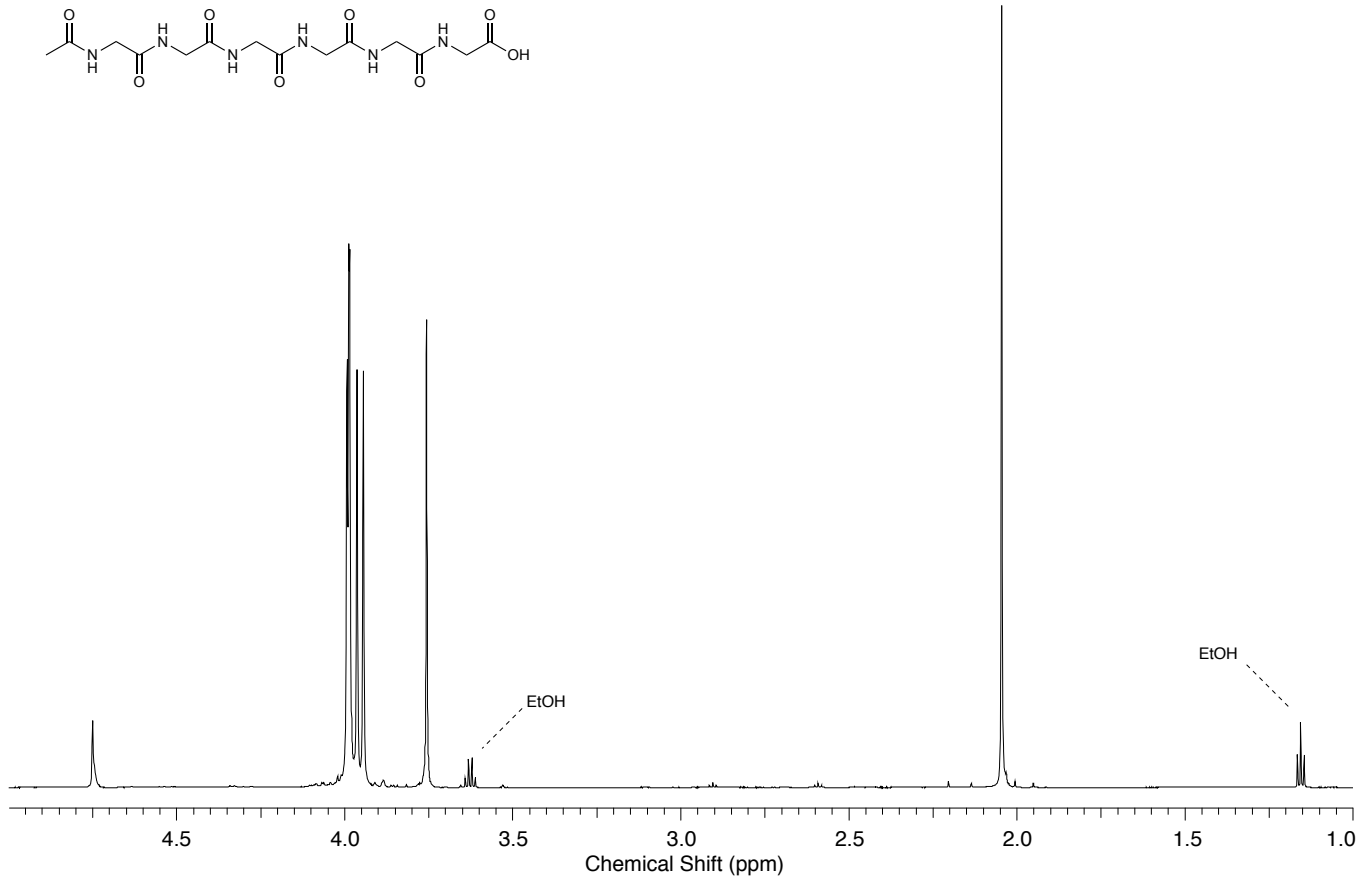


Fig. S184. ^{1}H NMR (700 MHz, D_2O, noesygppr1d, 1.0–5.0 ppm) spectrum to show **Ac-Gly-Gly-Gly-Gly-Gly-Gly-OH** isolated after precipitation by ethanol from the crude mixture obtained from the reaction of *N*-acetylglycylglycylglycine nitrile(**Ac-Gly$_3$-CN**; 100 mM), glycylglycylglycine (**Gly-Gly-Gly-OH**; 100 mM) and 3-mercaptopropionic acid (**MPA**; 160 mM) in H_2O (1 mL) at pH 7 at 60 °C.

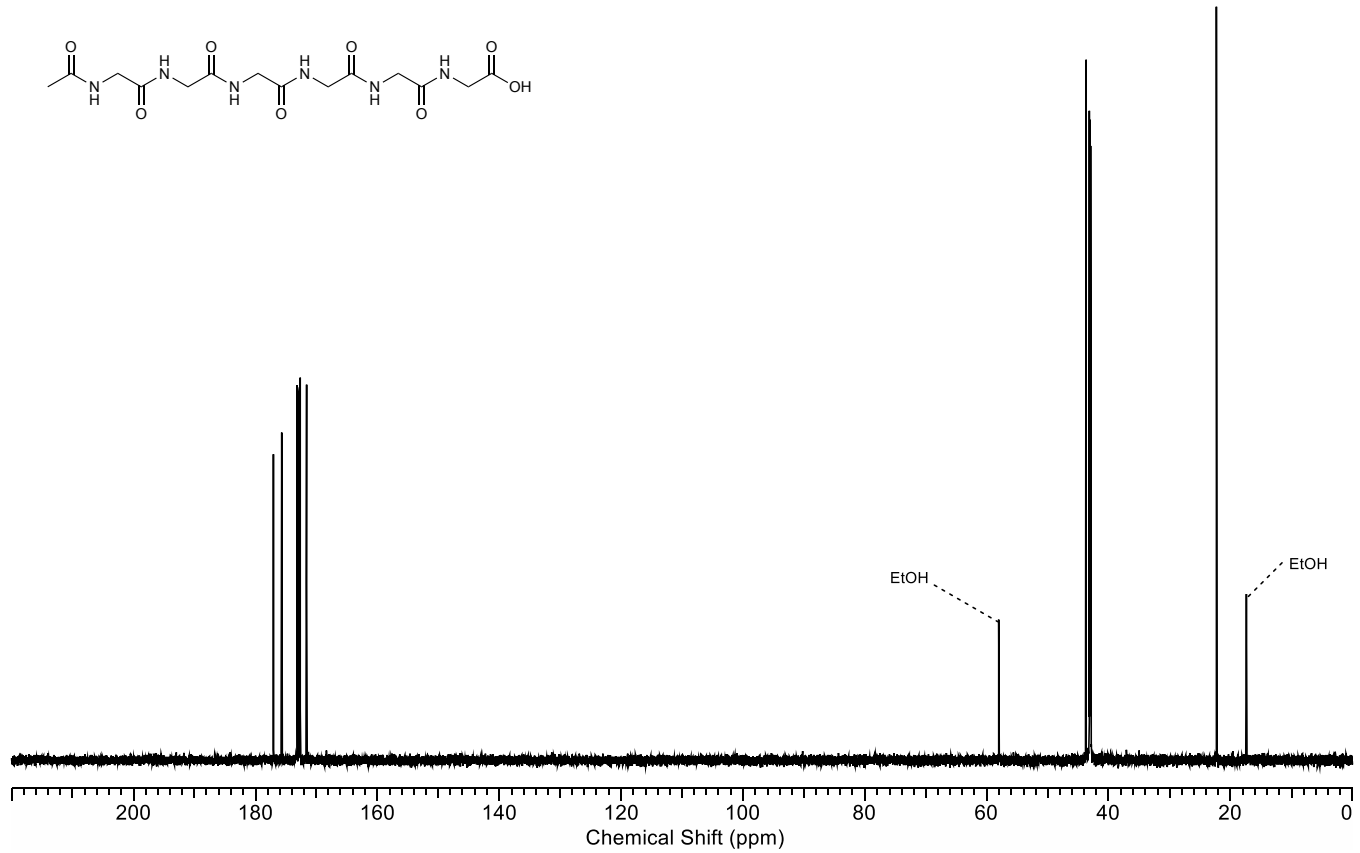


Fig. S185. ^{13}C NMR 176 MHz, D_2O, 0–220 ppm) spectrum to show **Ac-Gly-Gly-Gly-Gly-Gly-Gly-OH** isolated after precipitation by ethanol from the crude mixture obtained from the reaction of *N*-acetylglycylglycylglycine nitrile(**Ac-Gly$_3$-CN**; 100 mM), glycylglycylglycine (**Gly-Gly-Gly-OH**; 100 mM) and 3-mercaptopropionic acid (**MPA**; 160 mM) in H_2O (1 mL) at pH 7 at 60 °C.

N-Acetylglycylglycylglycylglycylglycylglycylglycine ***Ac-Gly-Gly-Gly-Gly-Gly-Gly-OH*** ^{1}H NMR (700 MHz, D_2O) (Fig. S184) δ 3.99 (s., 2H, Gly-(C2)–H_2), 3.99 (s, 2H, Gly-(C2)–H_2), 3.99 (s, 2H, Gly-(C2)–H_2), 3.96 (s, 2H, Gly-(C2)–H_2), 3.95 (s, 2H, Gly-(C2)–H_2), 3.76 (s, 2H, Gly-(C2)–H_2), 2.05 (s, 3H, $COCH_3$). ^{13}C NMR (176 MHz, D_2O) (Fig. S185) δ 177.0 (Gly-(C1)), 175.7 (**C**OCH_3), 173.1 (Gly-(C1)), 172.9 (Gly-(C1)), 172.8 (Gly-(C1)), 172.6 (Gly-(C1)), 171.6 (Gly-(C1)), 43.7 (Gly-(C2)), 43.2 (Gly-(C2)), 43.1 (Gly-(C2)), 43.0 (Gly-(C2)), 43.0 (Gly-(C2)), 42.9 (Gly-(C2)), 22.3 (CO**C**H_3).

N-Acetylglycylglycylglycylglycyl-L-alanylglycine ***Ac-Gly-Gly-Gly-Gly-Ala-Gly-OH***

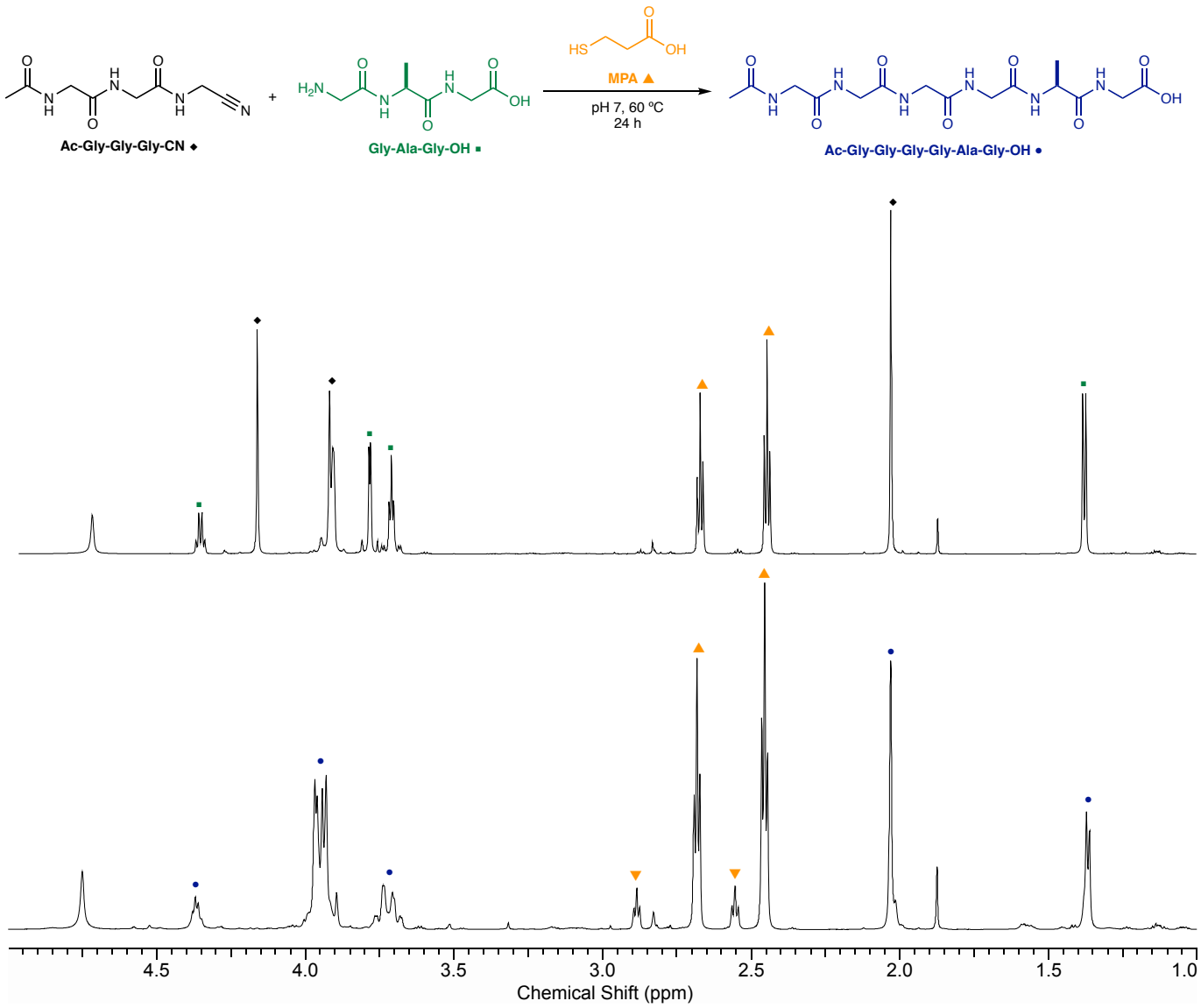


Fig. S186. ^{1}H NMR (700 MHz, H_2O, noesygppr1d, 1.0–5.0 ppm) spectrum to show the reaction of *N*-acetylglycylglycylglycine nitrile(**Ac-Gly$_3$-CN**; 100 mM), glycyl-L-alanylglycine (**Gly-Ala-Gly-OH**; 100 mM) and 3-mercaptopropionic acid (**MPA**; 160 mM) in H_2O (1 mL) at pH 7 at 60 °C. ▼ = 2-Carboxyethyldisulfide (formed by aerial oxidation of **MPA** ▲). Top = ^{1}H NMR spectrum acquisition before the reaction was heated 60 °C. Bottom = ^{1}H NMR spectrum acquisition after the reaction was heated 60 °C for 24 h.

N-Acetylglycylglycylglycylglycyl-L-alanylglycine ***Ac-Gly-Gly-Gly-Gly-Ala-Gly-OH*** (●) ^{1}H NMR (700 MHz, H_2O, noesygppr1d) (Fig. S186b) δ 4.36 (br. q, *J* = 7.1 Hz, 1H, Ala-(C2)−H), 3.97 (s, 2H, Gly$_1$-(C2)−H$_2$), 3.96 (s, 2H, Gly$_1$-(C2)−H$_2$), 3.94 (s, 2H, Gly$_1$-(C2)−H$_2$), 3.93 (s, 2H, Gly$_1$-(C2)−H$_2$), 3.78 – 3.72 (m, 1H, Gly$_6$-(C2)−H$_a$), 3.72 – 3.66 (m, 1H, Gly$_6$-(C2)−H$_b$), 2.01 (s, 3H, COCH$_3$), 1.37 (d, *J* = 7.1 Hz, 3H, Ala-(C2)−H$_3$).

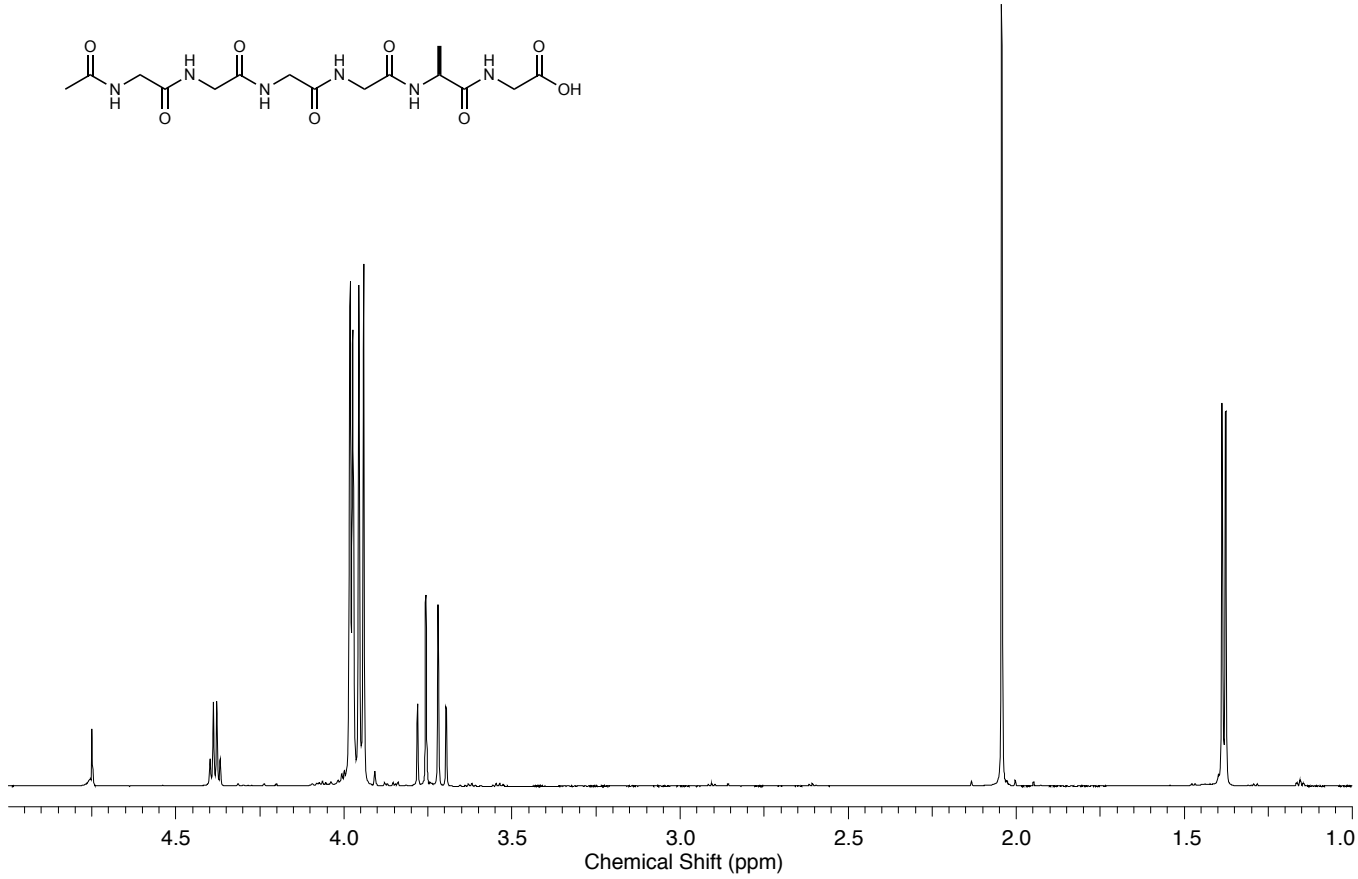


Fig. S187. ^{1}H NMR (700 MHz, D_2O, noesygppr1d, 1.0–5.0 ppm) spectrum to show **Ac-Gly-Gly-Gly-Gly-Ala-Gly-OH** isolated by the addition of acetone to the the crude mixture obtained from the reaction of *N*-acetylglycylglycylglycine nitrile(**Ac-Gly$_3$-CN**; 100 mM), glycyl-L-alanylglycine (**Gly-Ala-Gly-OH**; 100 mM) and 3-mercaptopropionic acid (**MPA**; 160 mM) in H_2O (1 mL) at pH 7 at 60 °C

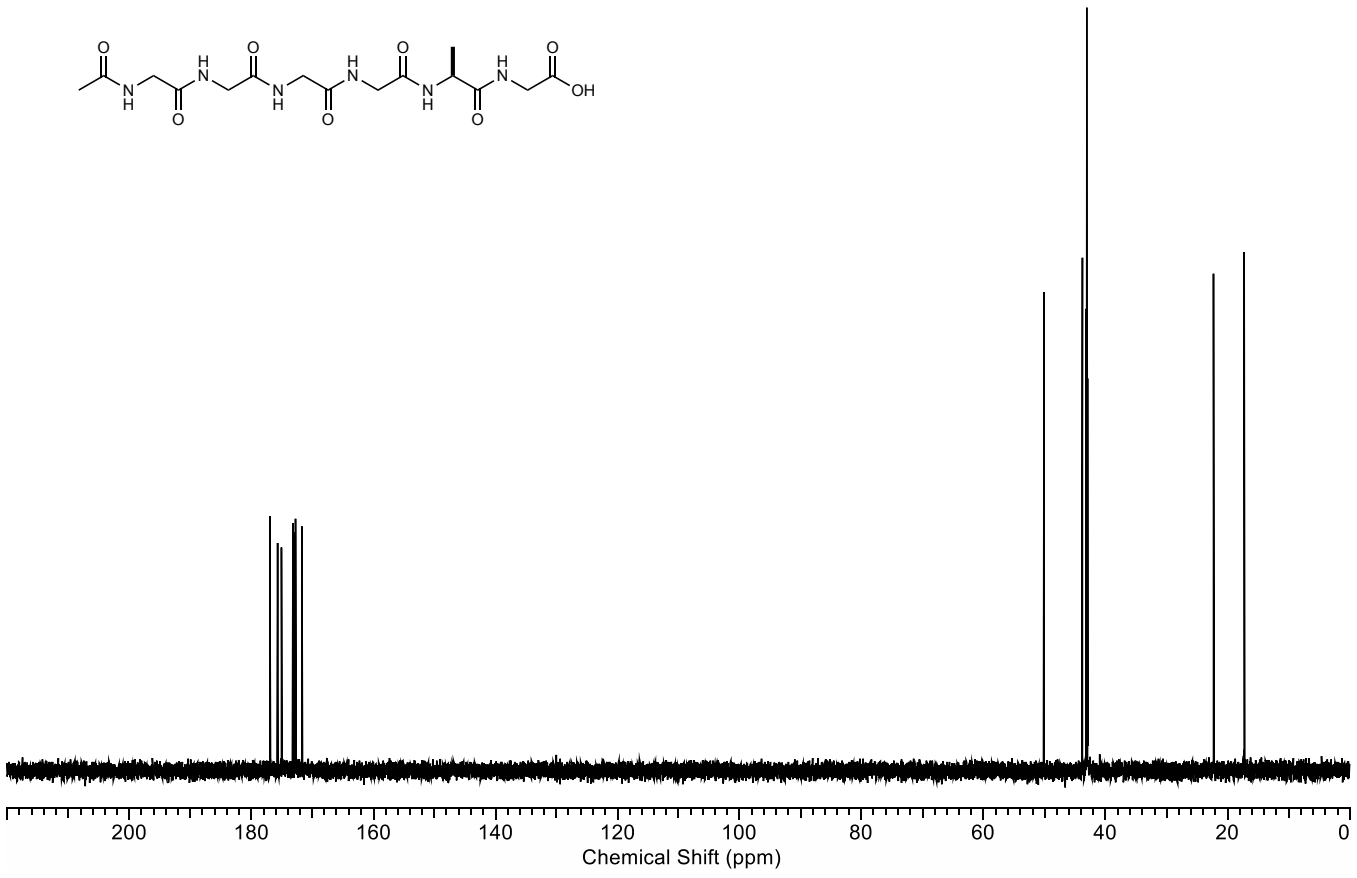
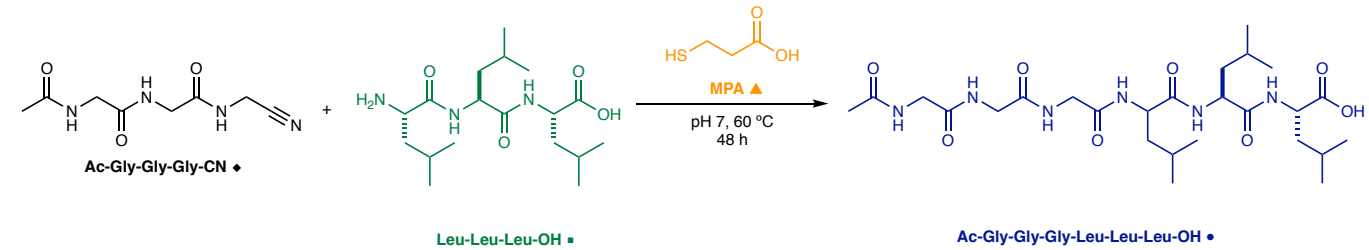


Fig. S188. ^{13}C NMR (176 MHz, D_2O, noesygppr1d, 0–220 ppm) spectrum to show **Ac-Gly-Gly-Gly-Gly-Ala-Gly-OH** isolated by the addition of acetone to the the crude mixture obtained from the reaction of *N*-acetylglycylglycylglycine nitrile(**Ac-Gly$_3$-CN**; 100 mM), glycyl-L-alanylglycine (**Gly-Ala-Gly-OH**; 100 mM) and 3-mercaptopropionic acid (160 mM) in H_2O (1 mL) at pH 7 at 60 °C.

N-Acetylglycylglycylglycylglycyl-L-alanylglycine ***Ac-Gly-Gly-Gly-Gly-Ala-Gly-OH*** ^{1}H NMR (700 MHz, D_2O, noesygppr1d) (Fig. S187) δ 4.35 (q, *J* = 7.2 Hz, 1H, Ala-(C2)−H), 3.95 (s, 2H, Gly_1-(C2)−H_2), 3.94 (s, 2H, Gly_1-(C2)−H_2), 3.93 (s, 2H, Gly_1-(C2)−H_2), 3.91 (s, 2H, Gly_1-(C2)−H_2), 3.74 (d, *J* = 17.2 Hz, 1H, Gly_6-(C2)−H_a), 3.68 (d, *J* = 17.2 Hz, 1H, Gly_6-(C2)−H_b), 2.01 (s, 3H, $COCH_3$), 1.35 (d, *J* = 7.2 Hz, 3H, Ala-(C2)−H_3). ^{13}C NMR (176 MHz, D_2O, noesygppr1d) (Fig. S188) δ 176.9 (Gly_6-(C1)), 175.7 (**C**OCH_3), 175.0 (Ala-(C1)), 173.1 (Gly_1-(C1)), 172.9 (Gly-(C1)), 172.7 (Gly-(C1)), 171.7 (Gly-(C1)), 50.1 (Ala-(C2)), 43.8 (Gly-(C2)), 43.2 (Gly-(C2)), 43.0 (Gly-(C2)), 43.0 (Gly-(C2)), 41.0 (Gly-(C2)), 22.3 (CO**C**H_3), 17.3 (Ala-(C3)).

N-Acetylglycylglycylglycyl-DL-leucyl-L-leucyl-L-leucine ***Ac-Gly-Gly-Gly-Leu-Leu-Leu-OH***[a]



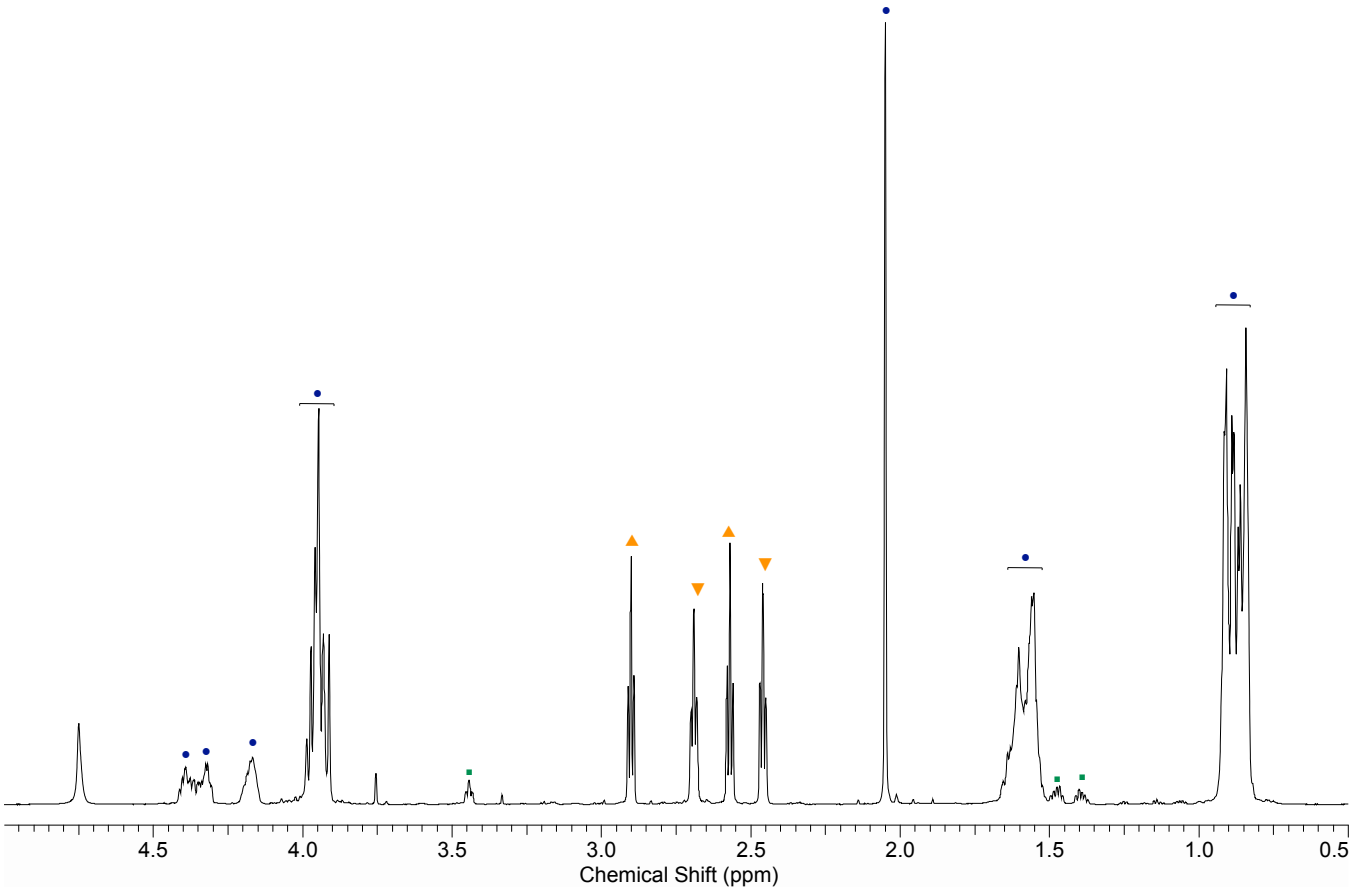


Fig. S189. ^{1}H NMR (700 MHz, H_2O, noesygppr1d, 0.5–5.0 ppm, pH 9.5) spectrum to show the reaction of *N*-acetylglycylglycylglycine nitrile(**Ac-Gly$_3$-CN**; 100 mM), leucinylleucinylleucine **Leu-Leu-Leu-OH**; 100 mM) and 3-mercaptopropionic acid (**MPA**; 160 mM) in H_2O (1 mL) at pH 7 at 60 °C after 48 h. ▼ = 2-Carboxyethyldisulfide (formed by aerial oxidation of **MPA** ▲).

^{1}H NMR (700 MHz, H_2O, noesygppr1d) *N-Acetylglycylglycylglycyl-DL-leucyl-L-leucyl-L-leucine* ***Ac-Gly-Gly-Gly-Leu-Leu-Leu-OH*** (●): (2 diastereoisomers) δ 4.35 - 4.26 (m, 2H, Leu-(C2)−H), 4.26 - 4.20 (m, 2H, Leu-(C2)−H), 4.13 - 4.02 (m, 2H, Leu-(C2)−H), 3.92 - 3.80 (m, 12H, 3 × Gly-(C2)−H_2), 1.96 (s, 6H, 2 × $COCH_3$), 1.59 - 1.41 (m, 18H, 3 × Leu-(C3)−H_2 , 3 × Leu-(C4)−H_2), 0.87 - 0.67 (m, 36H, 3 × Leu-(C5)−H_3, 3 × Leu-(C5)−H_3′).

[a] NMR analysis of the starting reaction mixture was not carried out before heating at 60 ºC due to the limited solubility of **Leu-Leu-Leu-OH** at pH 7. The reaction mixture was, however, homogenous after 48 h due to the conversion of **Leu-Leu-Leu-OH** to **Ac-Gly-Gly-Gly-Leu-Leu-Leu-OH**, the latter exhibiting greater solubility at pH 7. The reaction was adjusted from pH 7.0 to 9.5 prior to final NMR analysis to ensure any residual **Leu-Leu-Leu-OH** was accounted for.

N-Acetylglycylglycylglycylglyclglycyl-L-histidine ***Ac-Gly-Gly-Gly-Gly-Gly-His-OH***

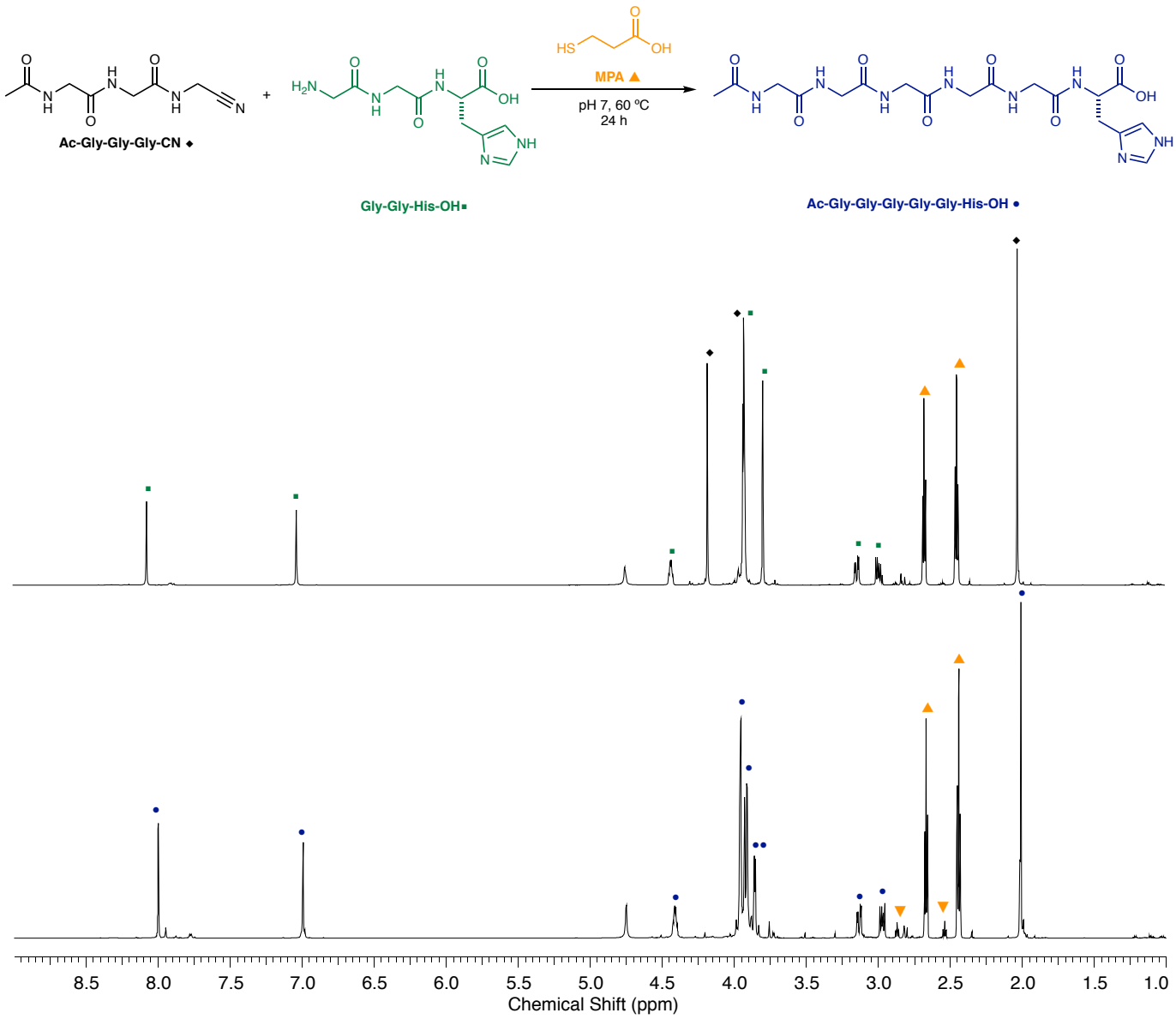


Fig. S190. ^{1}H NMR (700 MHz, H_2O, noesygppr1d, 1.0-9.0 ppm) spectrum to show the reaction of *N*-acetylglycylglycylglycine nitrile(**Ac-Gly$_3$-CN**; 100 mM), glycylglycyl-L-histidine (**Gly-Gly-His-OH**; 100 mM) and 3-mercaptopropionic acid (**MPA**; 160 mM) in H_2O (1 mL) at pH 7 at 60 °C. ▼ = 2-Carboxyethyldisulfide (formed by aerial oxidation of **MPA** ▲). Top = ^{1}H NMR spectrum acquisition before the reaction was heated 60 °C. Bottom = ^{1}H NMR spectrum acquisition after the reaction was heated 60 °C for 24 h.

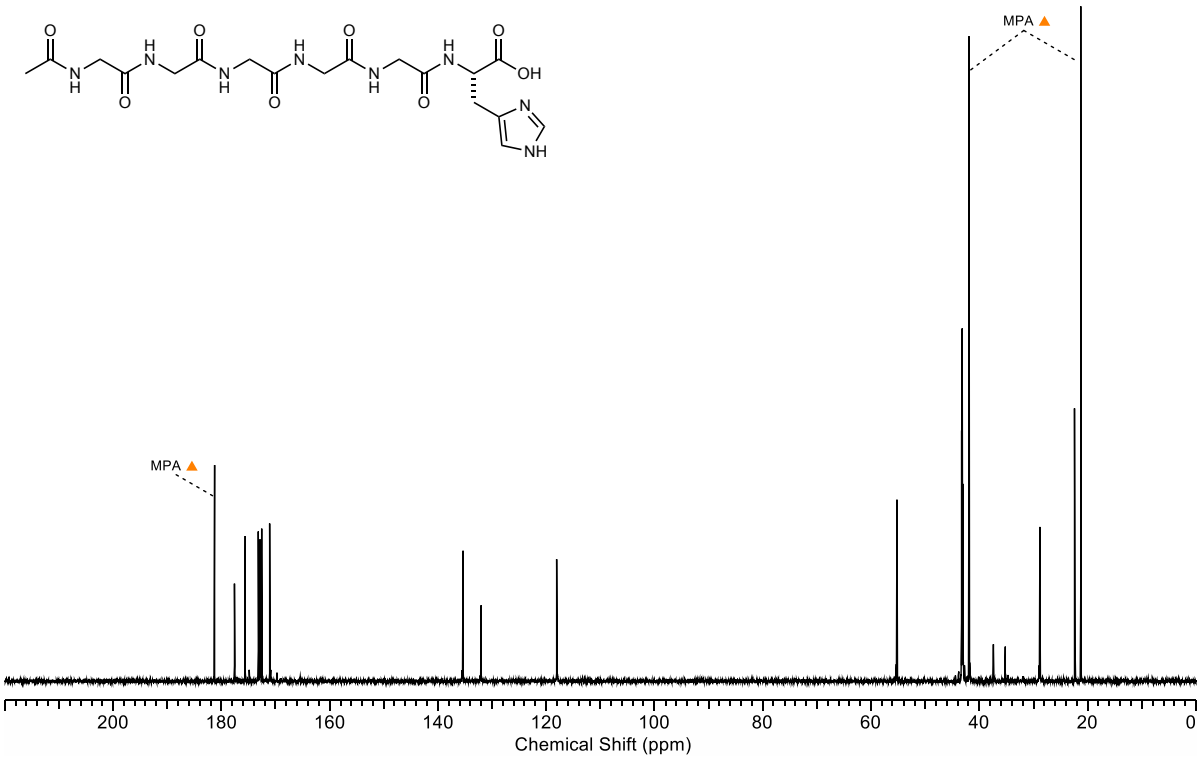


Fig. S191. ^{13}C NMR (176 MHz, H_2O, noesygppr1d, 0–220 ppm) spectrum to show the reaction of *N*-acetylglycylglycylglycine nitrile(**Ac-Gly$_3$-CN**; 100 mM), glycylglycyl-L-histidine (**Gly-Gly-His-OH**; 100 mM) and 3-mercaptopropionic acid (**MPA**; 160 mM) in H_2O (1 mL) at pH 7 at 60 °C. ▼ = 2-Carboxyethyldisulfide (formed by aerial oxidation of **MPA** ▲).

N-Acetylglycylglycylglycylglycylglycyl-L-histidine ***Ac-Gly-Gly-Gly-Gly-Gly-His-OH*** (•): ^{1}H NMR (700 MHz, H_2O, noesygppr1d, Fig. S190) δ 8.00 (s, 1H, His-(C2′)−H), 6.90 (s, 1H, His-(C4′)−H), 4.44 - 4.37 (m, 1H, His-(C2)−H), 3.95 (br. s., 4H, Gly-(C2)−H_2 × 2), 3.93 (br. s., 2H, Gly-(C2)−H_2), 3.91 (br. s., 2H, Gly-(C2)−H_2), 3.87 (AB, *J* = 17.2 Hz, 1H, Gly-(C2)−H), 3.84 (AB, *J* = 17.2 Hz, 1H, Gly-(C2)−H), 3.13 (ABX, *J* = 4.5, 15.0 Hz, 1H, His-(C3)−H), 2.97 (ABX, *J* = 8.4, 15.0 Hz, 1H, His-(C3′)−H), 2.02 (s, 3H, $COCH_3$). ^{13}C NMR (176 MHz, H_2O, noesygppr1d, Fig. S191) δ 177.5 (His-(C1)), 175.6 (**C**OCH_3), 173.1 (Gly-(C1)), 172.9 (Gly-(C1)), 172.7 (Gly-(C1)), 172.5 (Gly-(C1)), 171.1 (Gly$_5$-(C1)), 135.3 ((His-(C2′)), 132.0 (His-(C4′)), 118.0 (His-(C5′)), 55.2 (His-(C2)), 43.3 (Gly-(C2)), 43.2 (Gly-(C2)), 43.16 (Gly-(C2)), 43.15 (Gly-(C2)), 43.0 (Gly-(C2)), 28.8 (His-(C3)), 22.4 (CO**C**H_3).

N-Acetylglycylglycylglycyl-DL-methionyl-L-alanyl-L-serine **Ac-Gly-Gly-Gly-Met-Ala-Ser-OH**

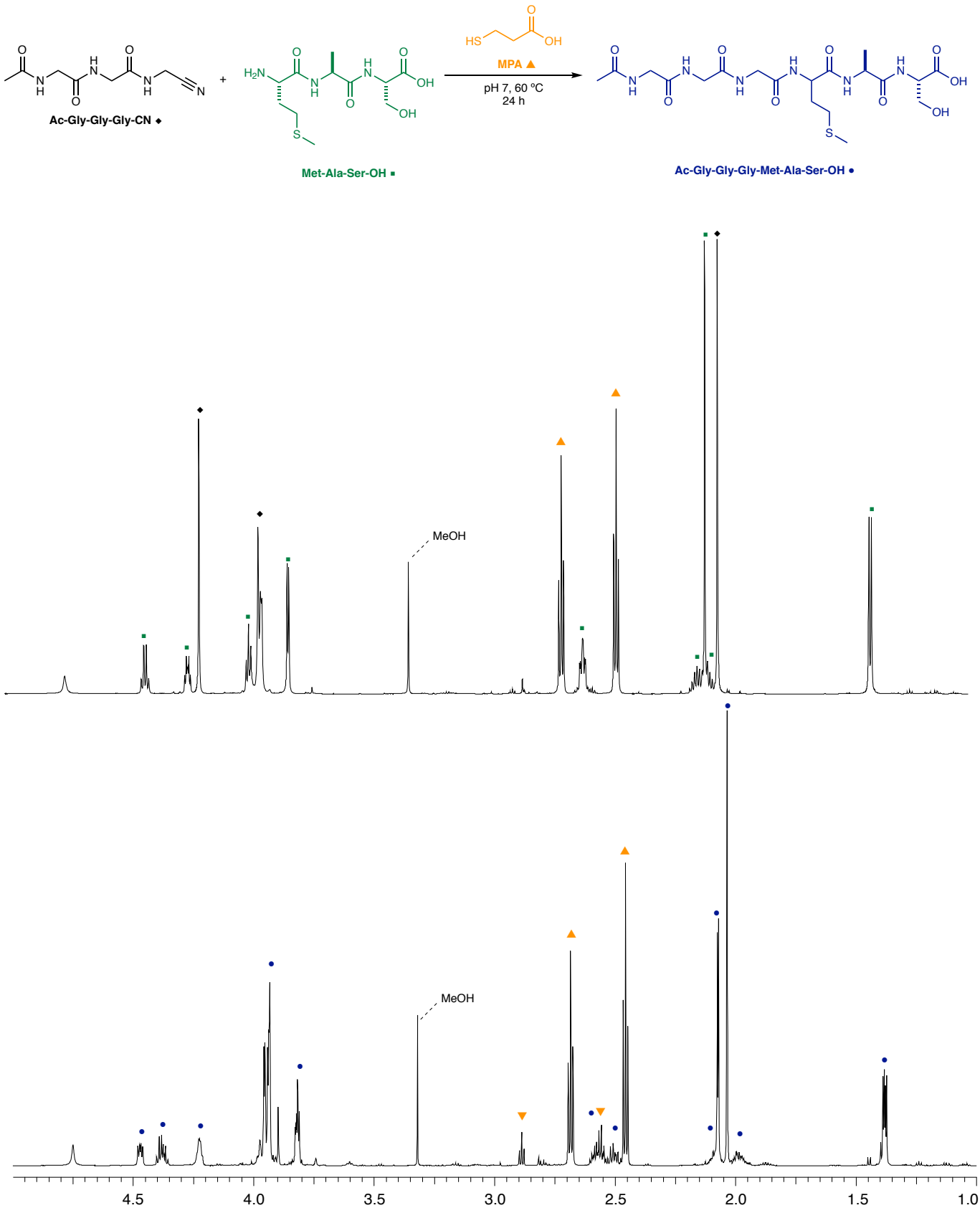


Fig. S192. ^{1}H NMR (700 MHz, H_2O, noesygppr1d, 1.0–5.0 ppm) spectrum to show the reaction of *N*-acetylglycylglycylglycine nitrile(**Ac-Gly$_3$-CN**; 100 mM), L-methionyl-L-alanyl-L-serine (**Met-Ala-Ser-OH**; 100 mM) and 3-mercaptopropionic acid (**MPA**; 160 mM) in H_2O (1 mL) at pH 7 at 60 °C. ▼ = 2-Carboxyethyldisulfide (formed by aerial oxidation of **MPA** ▲). Top = ^{1}H NMR spectrum acquisition before the reaction was heated 60 °C. Bottom = ^{1}H NMR spectrum acquisition after the reaction was heated 60 °C for 24 h.

N-Acetylglycylglycylglycyl-DL-methionyl-L-alanyl-L-serine ***Ac-Gly-Gly-Gly-Met-Ala-Ser-OH*** (•) (2 diastereoisomers): ^{1}H NMR (700 MHz, H_2O, noesygppr1d, Fig. S192b) δ 4.47 (dd, *J* = 5.2, 9.0 Hz, 2H, Met-(C2)–H), 4.41 - 4.34 (m, 2H, Ala-(C2)–H), 4.26 - 4.19 (br. m, 2H, Ser-(C2)–H), 3.97 - 3.91 (m, 12H, Gly_1-(C2)–H_2, Gly_2-(C2)–H_2, Gly_3-(C2)–H_2), 3.84 -3.79 (m, 4H, Ser-(C3)–H_2), 2.62 - 2.54 (m, 2H, Met-(C3)–H), 2.54 - 2.47 (m, 2H, Met-(C3)–H′), 2.11 - 2.05 (m, 8H, Met-(C4)–H), SCH_3), 2.04 (s, 6H, $COCH_3$), 2.02 - 1.95 (m, 2H, Met-(C4)–H′), 1.40 - 1.36 (m 6H, Ala-(C3)–H_2).

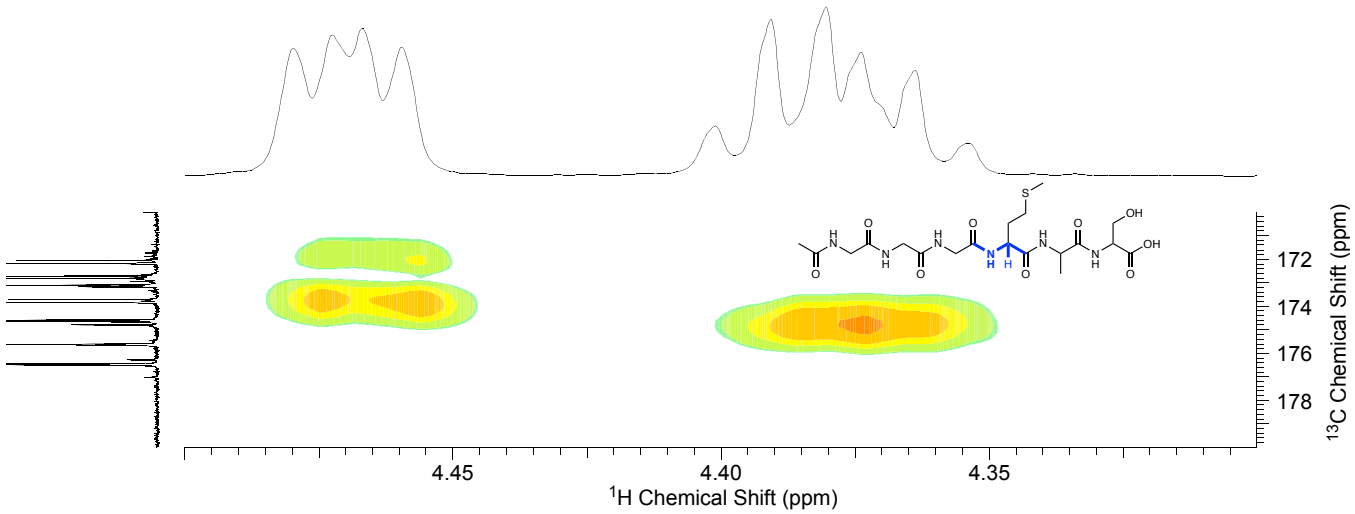


Fig. S193. ^{1}H–^{13}C HMBC (^{1}H: 700 MHz [4.40–4.50 ppm], ^{13}C: 176 MHz [170-180 ppm]) spectrum showing the diagnostic $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling of **Met**-(C2)–H in a diastereoisomeric mixture of **Ac-Gly-Gly-Gly-Met-Ala-Ser-OH** (δ_H = 4.47 ppm, δ_c = 173.8, 173.7, 172.2, 172.1 ppm). See Fig. S192b for expanded and labelled ^{1}H NMR spectrum.

N-Acetylglycylglycylglycyl-DL-phenylalanylglycylglycine ***Ac-Gly-Gly-Gly-Phe-Gly-Gly-OH***

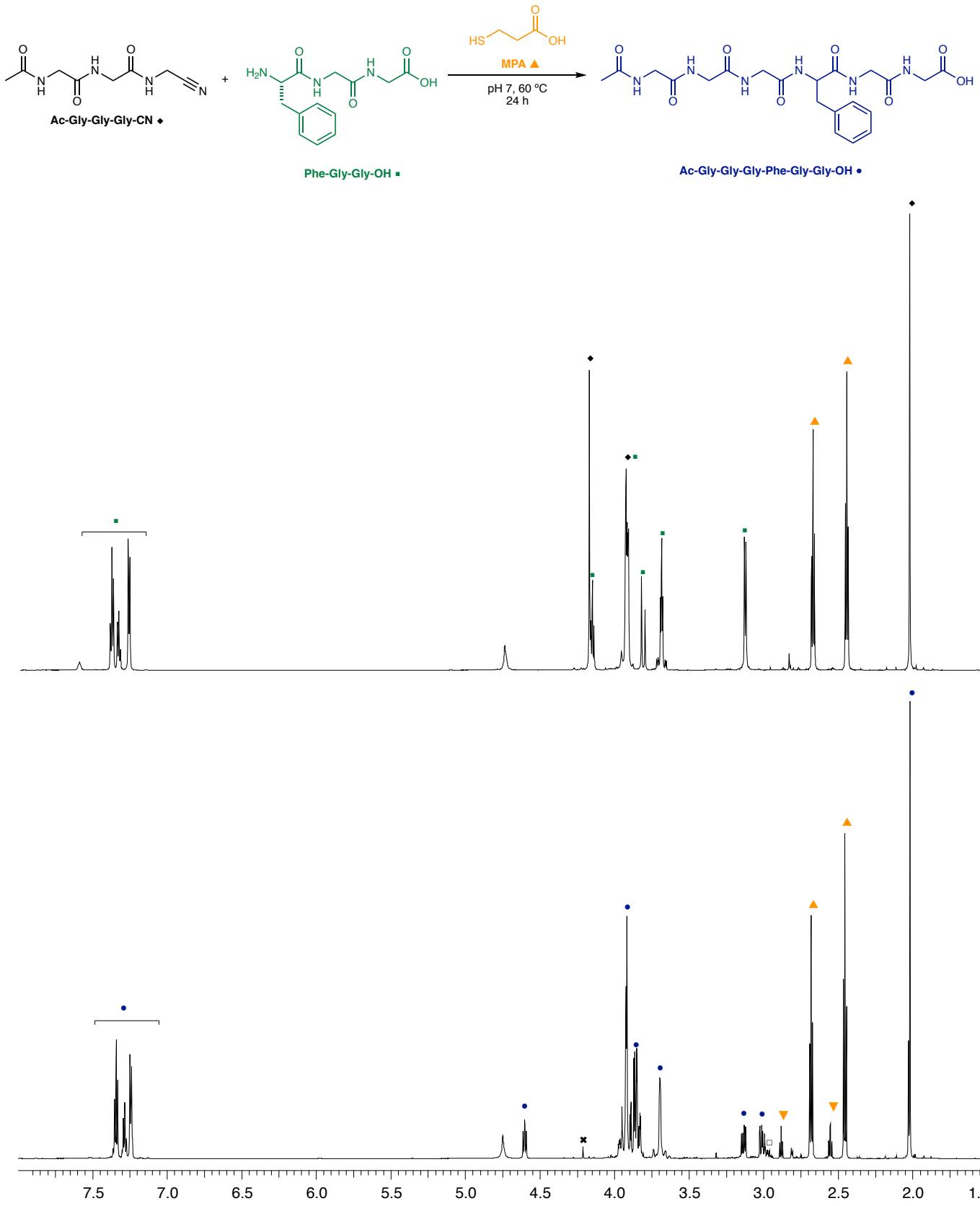


Fig. S194. ^{1}H NMR (700 MHz, H_2O, noesygppr1d, 1.5–8.0 ppm) spectrum to show the reaction of *N*-acetylglycylglycylglycine nitrile (**Ac-Gly$_3$-CN**; 100 mM), L-phenylalanylglycylglycine (**Phe-Gly-Gly-OH**; 100 mM) and 3-mercaptopropionic acid (**MPA**; 160 mM) in H_2O (1 mL) at pH 7 at 60 °C. ▼ = 2-Carboxyethyldisulfide (formed by aerial oxidation of **MPA** ▲). Top = ^{1}H NMR spectrum acquisition before the reaction was heated 60 °C. Bottom = ^{1}H NMR spectrum acquisition after the reaction was heated 60 °C for 24 h. **x** = **Ac-Gly$_2$-Gly^{N}-NH$_2$**. □ = **Ac-Gly-Gly-Gly^{N}-Phe-Gly-Gly-OH** (12%).

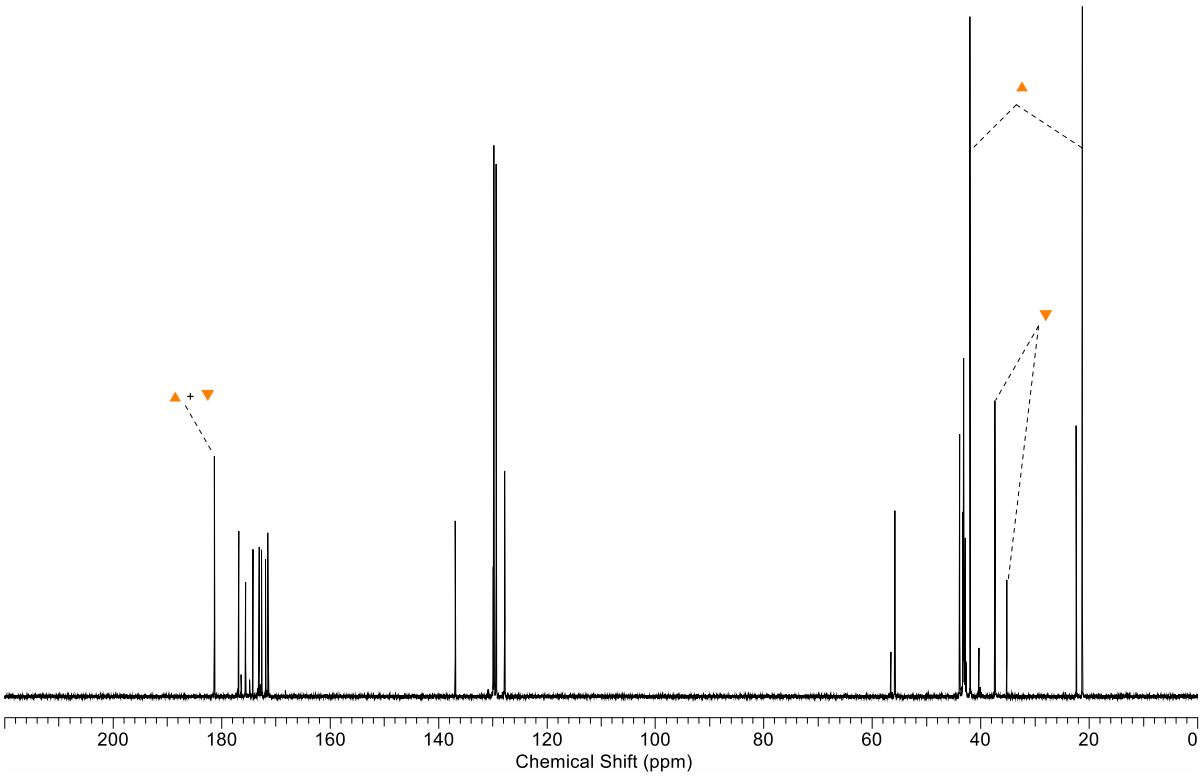
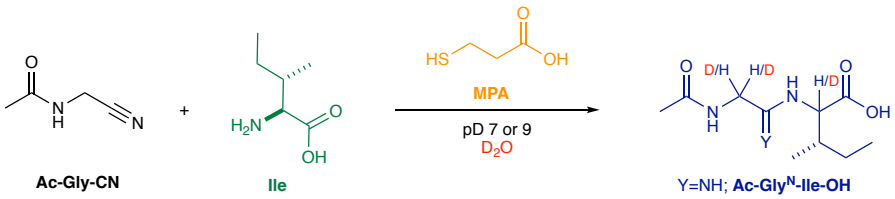


Fig. S195. ^{13}C NMR (176 MHz, H_2O, noesygppr1d, 0–220 ppm) spectrum to show the reaction of *N*-acetylglycylglycylglycine nitrile (**Ac-Gly$_3$-CN**; 100 mM), L-phenylalanylglycylglycine (**Phe-Gly-Gly-OH**; 100 mM) and 3-mercaptopropionic acid (**MPA**; 160 mM) in H_2O (1 mL) at pH 7 at 60 °C. ▼ = 2-Carboxyethyldisulfide (formed by aerial oxidation of **MPA** ▲).

^{1}H NMR (700 MHz, D_2O, noesygppr1d, Fig. S194b) *N-Acetylglycylglycylglycyl-DL-phenylalanylglycylglycine* ***Ac-Gly-Gly-Gly-Phe-Gly-Gly-OH*** (•) δ 7.37 - 7.32 (m, 4H, Ar), 7.31 - 7.26 (m, 2H, Ar), 7.25 (d, *J* = 7.0 Hz, 4H, Ar), 4.60 (app. t, *J* = 7.4 Hz, 2H, Phe-(C2)–H), 3.92 (br. s., 4H, Gly-(C2)–H), 3.92 (br. s., 4H, Gly-(C2)–H), 3.89 - 3.84 (m, 8H, 2 × Gly-(C2)–H), 3.69 (br. s., 4H, Gly-(C2)–H), 3.14 (ABX, *J* = 6.8, 13.0 Hz, 2H, Phe-(C3)–H′), 3.01 (ABX, *J* = 8.3, 13.0 Hz, 2H, Phe-(C3)–H′′), 2.02 (s, 6H, $COCH_3$). ^{13}C NMR (176 MHz, H_2O, noesygppr1d, Fig. S195) (2 diastereoisomers) δ 176.9 (CO), 175.6 (CO), 174.2 (CO), 173.1 (CO), 172.6 (CO), 171.9 (CO), 171.5 (CO), 136.9 (Ar), 129.8 (Ar), 129.4 (Ar), 127.8 (Ar), 55.9 (Phe-(C2)), 43.9 (2 × Gly-(C2)), 43.3 (2 × Gly-(C2)), 43.2 (2 × Gly-(C2)), 42.9 (4 × Gly-(C2)), 37.4 (Phe-(C3)), 22.4 ($CO\mathbf{C}H_3$).

Peptide deuteration studies

*Coupling of N-acetylglycine nitrile **Ac-Gly-CN** with L-isoleucine **Ile** in D_2O*

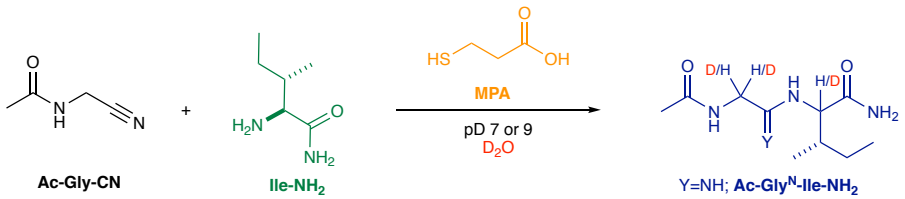


N-Acetylglycine nitrile **Ac-Gly-CN** (100 mM) and L-isoleucine **Ile** (100 mM) and 3-mercaptopropionic acid (**MPA**; 200 mM) were dissolved in D_2O (1 mL) and the solution was adjusted to pD 7.0 or 9.0 with 4 M NaOD and incubated at room temperature or 60 °C. The reaction was cooled to room temperature and analysed by NMR spectroscopy (^1H; ^{13}C; ^1H–^1H COSY; ^1H–^{13}C HSQC; ^1H–^{13}C HMBC). Observations are summarised in Table S9.

Entry	Time (d)	pH	Temp (°C)	∑(yield, %) **Ac-Gly$_{H/D}$-Ile$_{H/D}$-OH** + **Ac-Gly$^N_{H/D}$-Ile$_{H/D}$-OH**	Comment
1	10	7	rt	88	<5% Ile-(C2)-deuteration 12% Gly-(C2) deuteration
2	1	7	60	80	35% Ile-(C2) deuteration >95% Gly-(C2) deuteration
3	3	9	rt	>95	<5% Ile-(C2) deuteration >95% Gly-(C2) deuteration
4	1	9	60	n.d	>90% Ile-(C2)-deuteration >90% Gly-(C2)-deuteration

Table S9. Yields and deuteration levels of the coupling product (**Ac-Gly$_{H/D}$-Ile$_{H/D}$-OH** and **Ac-Gly$^N_{H/D}$-Ile$_{H/D}$-OH**) produced by 3-mercaptopropionic acid **MPA** (200 mM) catalysed coupling of **Ac-Gly-CN** (100 mM) with L-isoleucine **Ile** (100 mM) in D_2O at pD 7 or 9, and at room temperature or 60 °C. n.d = not determined due to extensive deuteration. rt = room temperature.

*Coupling of N-acetylglycine nitrile **Ac-Gly-CN** with L-isoleucinamide **Ile-NH₂** in D_2O*

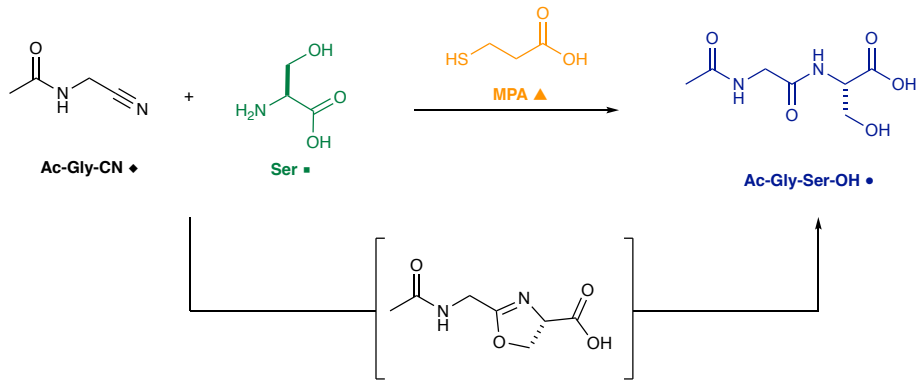


N-Acetylglycine nitrile **Ac-Gly-CN** (100 mM) and L-isoleucinamide **Ile-NH₂** (100 mM) and 3-mercaptopropionic acid (**MPA**; 200 mM) were dissolved in D_2O (1 mL) and the solution was adjusted to pD 7.0 or 9.0 with 4 M NaOD and incubated at room temperature or 60 °C. The reaction was cooled to room temperature and analysed by NMR spectroscopy (^1H; ^{13}C; ^1H–^1H COSY; ^1H–^{13}C HSQC; ^1H–^{13}C HMBC). Observations are summarised in Table S10.

Entry	Time (d)	pH	Temp (°C)	Comment
1	8	7	rt	>90% Ile-(C2)-deuteration >90% Gly-(C2)-deuteration
2	1	7	60	>90% Ile-(C2)-deuteration >90% Gly-(C2)-deuteration
3	1	9	rt	>90% Ile-(C2)-deuteration >90% Gly-(C2)-deuteration
4	1	9	60	>90% Ile-(C2)-deuteration >90% Gly-(C2)-deuteration

Table S10. Deuteration levels of the coupling product (**Ac-Gly$_{H/D}$-Ile$_{H/D}$-NH₂**) produced by 3-mercaptopropionic acid **MPA** (200 mM) catalysed coupling of **Ac-Gly-CN** (100 mM) with L-isoleucinamide **Ile-NH₂** (100 mM) in D_2O at pD 7 or 9, and at room temperature or 60 °C. Yields not determined due to extensive deuteration. rt = room temperature.

*Coupling of N-acetylglycine nitrile **Ac-Gly-CN** with L-serine **Ser** in H_2O or D_2O*



N-Acetylglycine nitrile **Ac-Gly-CN** (200 mM) and L-serine **Ser** (200 mM) and 3-mercaptopropionic acid (**MPA**; 200 mM) were dissolved in H_2O or D_2O (1 mL). The solution was adjusted to pH 7.0 with 4 M NaOH, or pD 7.0 with 4 M NaOD, and incubated at 60 °C for 48 h. The reaction was cooled to room temperature and analysed by NMR spectroscopy (Fig. S196). Yield of **Ac-Gly-Ser-OH** and the amounts of deuteration are reported in Table S11.

Entry	Solvent	Ac-Gly-Ser-OH (%)	Σ Integral		Deuteration (%)
			t = 0	t = 48	
1	H_2O	74	5.00	4.86	2.80
2	D_2O	60	5.00	4.73	5.40

Table S11. Yields of **Ac-Gly-Ser-OH** produced by 3-mercaptopropionic acid **MPA** (200 mM) catalysed coupling of **Ac-Gly-CN** (100 mM) with L-serine **Ser** (200 mM) in H_2O or D_2O at pH or pD 7 after 48 h at 60 °C. **Σ Integral** is the total sum of integration in the (C2)–H and (C3)–H region of the ^{1}H NMR spectrum (3.5 – 4.5 ppm), using **MPA** and 2-carboxyethyldisulfide (the aerial oxidation product of **MPA**) as the internal standard, before (**t = 0**) and after heating at 60 °C for 48 h (**t = 48**). This value was further verified by the total integration of the $COCH_3$ region between 1.7 – 2.1 ppm of the ^{1}H NMR spectrum. The total integral intensity of the $COCH_3$ region after 48 h was completely recovered (>98%) in all cases. **Deuteration (%)** is the amount of (C2)–H and/or (C3)–H deuteration that was calculated by comparing the total ^{1}H NMR integral analysis of the (C2)–H and (C3)–H regions (3.5–4.5 ppm) before (**t = 0**) and after incubation of the reaction at 60 °C for 48 h (**t = 48**).

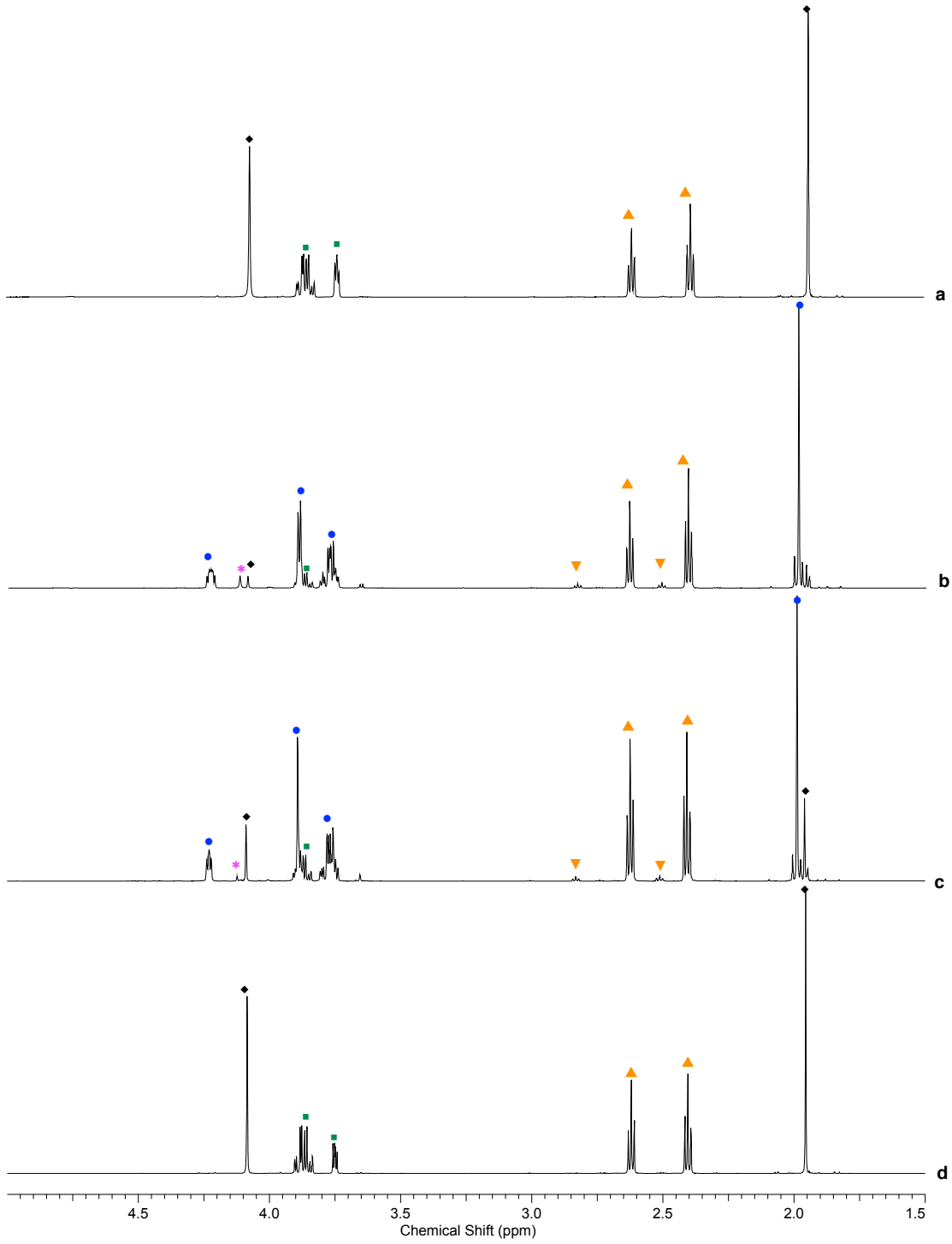
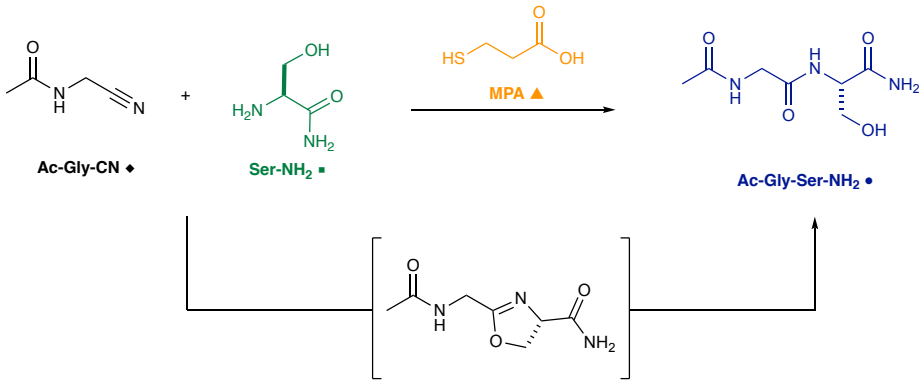


Fig. S196. ^{1}H NMR (600 MHz, noesygppr1d, 1.5–5.0 ppm) spectra to show the reaction of *N*-acetylglycine nitrile **Ac-Gly-CN** (200 mM) with L-serine **Ser** (200 mM) and 3-mercaptopropionic acid **MPA** (200 mM) after heating for 48 h at 60 °C in H_2O (pH 7) or D_2O (pD 7). a) **Ac-Gly-CN**, **Ser** and **MPA** in H_2O at pH 7 before heating at 60 °C. b) **Ac-Gly-CN**, **Ser** and **MPA** in H_2O at pH 7 after heating at 60 °C. c) **Ac-Gly-CN**, **Ser** and **MPA** in D_2O at pD 7 after heating at 60 °C. d) **Ac-Gly-CN**, **Ser** and **MPA** in D_2O at pD 7 before heating at 60 °C. ▼ = 2-Carboxyethyldisulfide (formed by aerial oxidation of **MPA** ▲). ✳ = *N*-(2-amino-2-iminoethyl)acetamide, **Ac-Gly^{N}-NH$_2$**.

Coupling of N-acetylglycine nitrile ***Ac-Gly-CN*** *with L-serinamide* ***Ser-NH₂*** *in H_2O or D_2O*



N-Acetylglycine nitrile **Ac-Gly-CN** (200 mM) and L-serinamide **Ser-NH₂** (200 mM) and 3-mercaptopropionic acid (**MPA**; 200 mM) were dissolved in H_2O or D_2O (1 mL). The solution was adjusted to pH 7.0 with 4 M NaOH, or pD 7.0 with 4 M NaOD, and incubated at 60 °C for 48 h. The reaction was cooled to room temperature and analysed by NMR spectroscopy (Fig. S197). Yield of **Ac-Gly-Ser-NH₂** and the amounts of deuteration are reported in Table S12.

Entry	Solvent	Ac-Gly-Ser-NH₂ (%)	Integral area		Deuteration (%)
			t = 0 h	t = 48 h	
1	H_2O	80	5.00	4.75	5.00
2	D_2O	80	5.00	4.55	9.00

Table S12. Yields of **Ac-Gly-Ser-NH₂** produced by 3-mercaptopropionic acid **MPA** (200 mM) catalysed coupling of **Ac-Gly-CN** (100 mM) with L-serinamide **Ser-NH₂** (200 mM) in H_2O or D_2O at pH or pD 7 after 48 h at 60 °C. **Σ Integral** is the total sum of integration in the (C2)–H and (C3)–H region of the ^{1}H NMR spectrum (3.5 – 4.5 ppm), using **MPA** and 2-carboxyethyldisulfide (the aerial oxidation product of **MPA**) as the internal standard, before (**t = 0**) and after heating at 60 °C for 48 h (**t = 48**). This value was further verified by the total integration of the $COCH_3$ region between 1.7 – 2.1 ppm of the ^{1}H NMR spectrum. The total integral intensity of the $COCH_3$ region after 48 h was completely recovered (>98%) in all cases. **Deuteration (%)** is the amount of (C2)–H and/or (C3)–H deuteration that was calculated by comparing the total ^{1}H NMR integral analysis of the (C2)–H and (C3)–H regions (3.5–4.5 ppm) before (**t = 0**) and after incubation of the reaction at 60 °C for 48 h (**t = 48**).

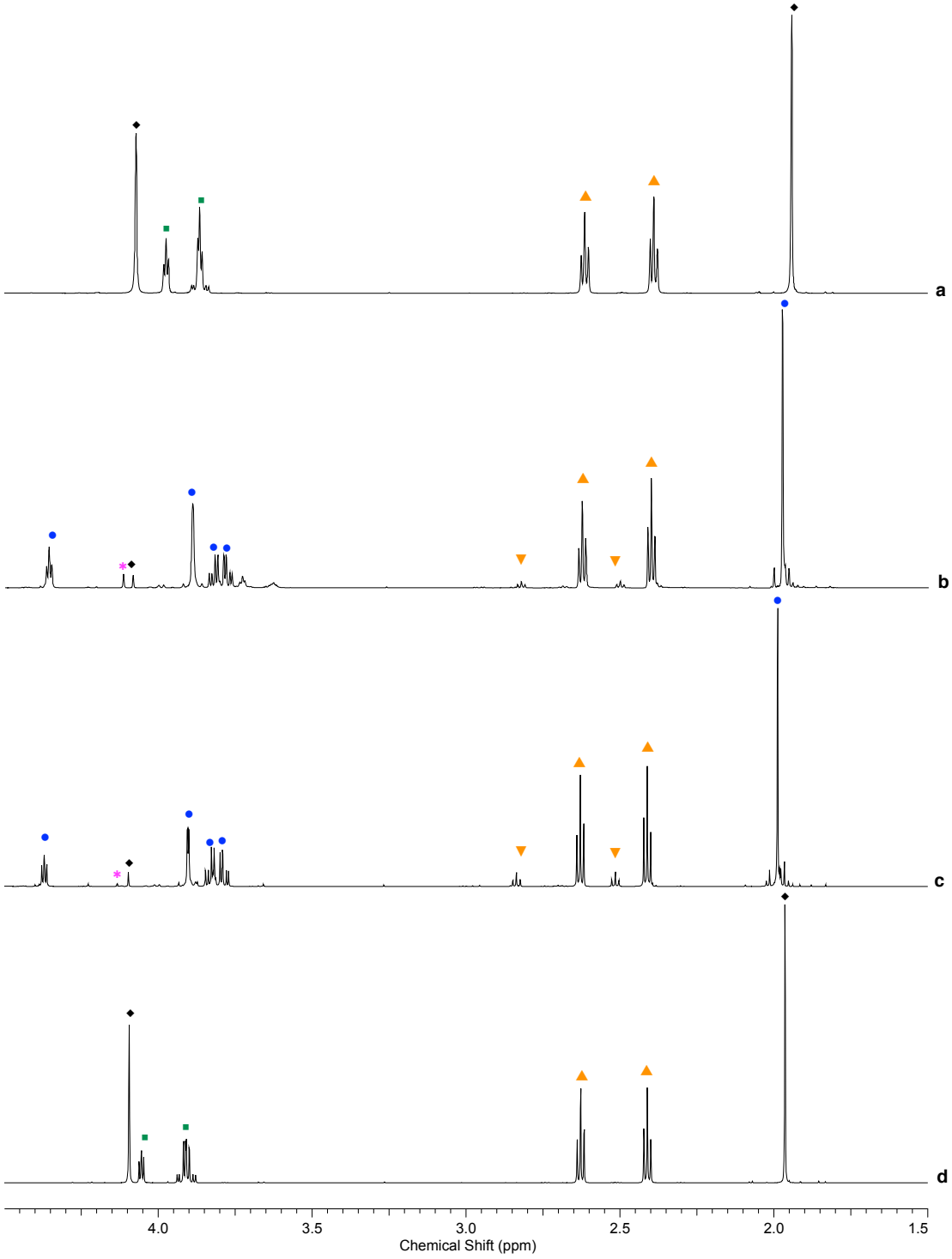
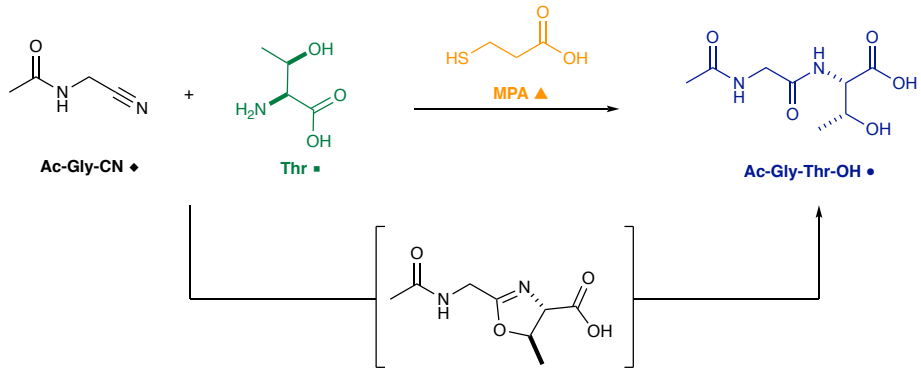


Fig. S197. ^{1}H NMR (600 MHz, noesygppr1d, 1.5–4.5 ppm) spectra to show the reaction of *N*-acetylglycine nitrile **Ac-Gly-CN** (200 mM) with L-serinamide **Ser-NH$_2$** (200 mM) and 3-mercaptopropionic acid **MPA** (200 mM) after heating for 48 h at 60 °C in H_2O (pH 7) or D_2O (pD 7). a) **Ac-Gly-CN**, **Ser-NH$_2$** and **MPA** in H_2O at pH 7 before heating at 60 °C. b) **Ac-Gly-CN**, **Ser-NH$_2$** and **MPA** in H_2O at pH 7 after heating at 60 °C. c) **Ac-Gly-CN**, **Ser-NH$_2$** and **MPA** in D_2O at pD 7 after heating at 60 °C. d) **Ac-Gly-CN**, **Ser-NH$_2$** and **MPA** in D_2O at pD 7 before heating at 60 °C. ▼ = 2-Carboxyethyldisulfide (formed by aerial oxidation of **MPA** ▲). ✳ = *N*-(2-amino-2-iminoethyl)acetamide, **Ac-Gly^{N}-NH$_2$**.

*Coupling of N-acetylglycine nitrile **Ac-Gly-CN** with L-threonine **Thr** in H_2O or D_2O*



N-Acetylglycine nitrile **Ac-Gly-CN** (200 mM) and L-threonine **Thr** (200 mM) and 3-mercaptopropionic acid (**MPA**; 200 mM) were dissolved in H_2O or D_2O (1 mL). The solution was adjusted to pH 7.0 with 4 M NaOH, or pD 7.0 with 4 M NaOD, and incubated at 60 °C for 48 h. The reaction was cooled to room temperature and analysed by NMR spectroscopy (Fig. S198). Yield of **Ac-Gly-Thr-OH** and the amounts of deuteration are reported in Table S13.

Entry	Solvent	Ac-Gly-Thr-OH (%)	Integral area		Deuteration (%)
			t = 0 h	t = 48 h	
1	H_2O	75	4.00	3.89	2.75
2	D_2O	80	4.00	3.51	12.25

Table S13. Yields of **Ac-Gly-Thr-OH** produced by 3-mercaptopropionic acid **MPA** (200 mM) catalysed coupling of **Ac-Gly-CN** (100 mM) with L-threonine **Thr** (200 mM) in H_2O or D_2O at pH or pD 7 after 48 h at 60 °C. **Σ Integral** is the total sum of integration in the (C2)–H and (C3)–H region of the ^{1}H NMR spectrum (3.5 – 4.5 ppm), using **MPA** and 2-carboxyethyldisulfide (the aerial oxidation product of **MPA**) as the internal standard, before (**t = 0**) and after heating at 60 °C for 48 h (**t = 48**). This value was further verified by the total integration of the $COCH_3$ region between 1.7 – 2.1 ppm of the ^{1}H NMR spectrum. The total integral intensity of the $COCH_3$ region after 48 h was completely recovered (>98%) in all cases. **Deuteration (%)** is the amount of (C2)–H and/or (C3)–H deuteration that was calculated by comparing the total ^{1}H NMR integral analysis of the (C2)–H and (C3)–H regions (3.5–4.5 ppm) before (**t = 0**) and after incubation of the reaction at 60 °C for 48 h (**t = 48**).

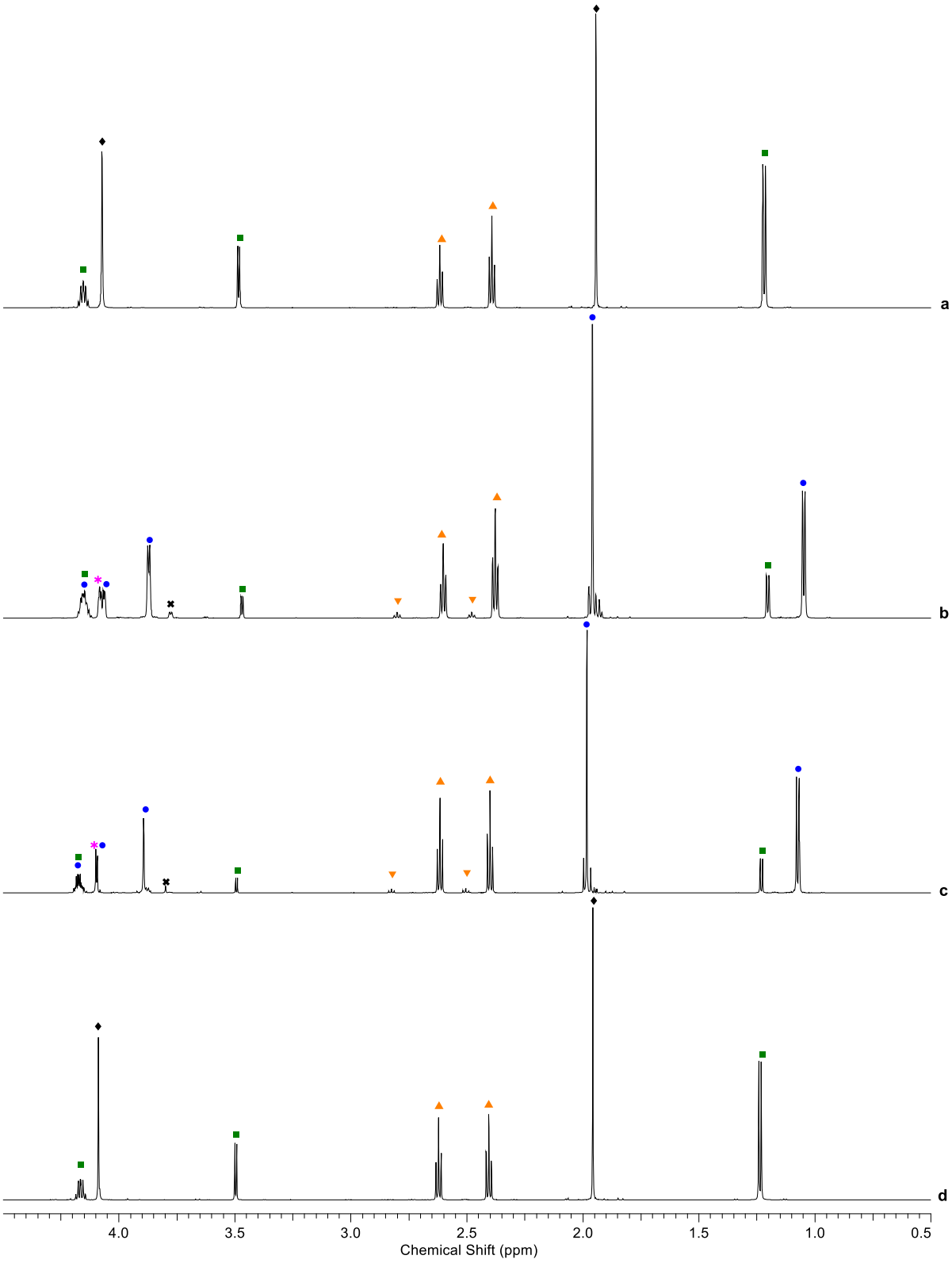
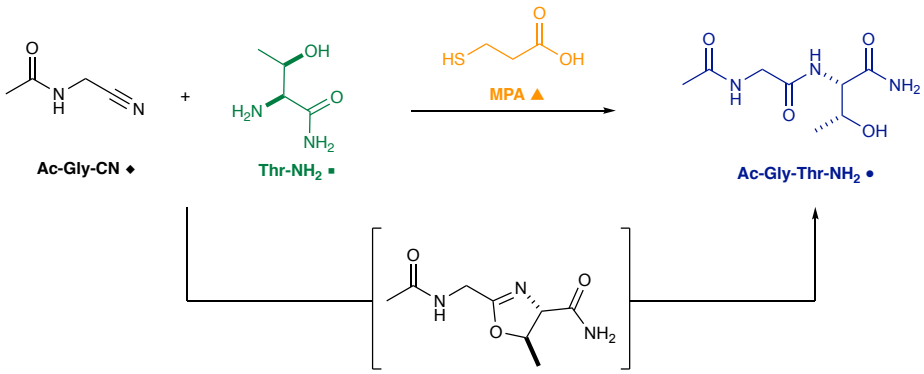


Fig. S198. ^{1}H NMR (600 MHz, noesygppr1d, 0.5–4.5 ppm) spectra to show the reaction of *N*-acetylglycine nitrile **Ac-Gly-CN** (200 mM) with L-threonine **Thr** (200 mM) and 3-mercaptopropionic acid **MPA** (200 mM) after heating for 48 h at 60 °C in H_2O (pH 7) or D_2O (pD 7). a) **Ac-Gly-CN**, **Thr** and **MPA** in H_2O at pH 7 before heating at 60 °C. b) **Ac-Gly-CN**, **Thr** and **MPA** in H_2O at pH 7 after heating at 60 °C. c) **Ac-Gly-CN**, **Thr** and **MPA** in D_2O at pD 7 after heating at 60 °C. d) **Ac-Gly-CN**, **Thr** and **MPA** in D_2O at pD 7 before heating at 60 °C. ▼ = 2-Carboxyethyldisulfide (formed by aerial oxidation of **MPA** ▲). ✳ = *N*-(2-amino-2-iminoethyl)acetamide, **Ac-Gly^{N}-NH$_2$**. × = *N*-acetylglycinamide, **Ac-Gly-NH$_2$**.

Coupling of N-acetylglycine nitrile ***Ac-Gly-CN*** *with L-threoninamide* ***Thr-NH₂*** *in H_2O or D_2O*



N-Acetylglycine nitrile **Ac-Gly-CN** (200 mM) and L-threoninamide **Thr-NH₂** (200 mM) and 3-mercaptopropionic acid (**MPA**; 200 mM) were dissolved in H_2O or D_2O (1 mL). The solution was adjusted to pH 7.0 with 4 M NaOH, or pD 7.0 with 4 M NaOD, and incubated at 60 °C for 48 h. The reaction was cooled to room temperature and analysed by NMR spectroscopy (Fig. S199). Yield of **Ac-Gly-Thr-NH₂** and the amounts of deuteration are reported in Table S14.

Entry	Solvent	Ac-Gly-Thr-NH₂ (%)	Integral area		Deuteration (%)
			t = 0 h	t = 48 h	
1	H_2O	85	4.00	3.92	2.00
2	D_2O	82	4.00	3.50	12.50

Table S14. Yields of **Ac-Gly-Thr-NH₂** produced by 3-mercaptopropionic acid **MPA** (200 mM) catalysed coupling of **Ac-Gly-CN** (100 mM) with L-threoninamide **Thr-NH₂** (200 mM) in H_2O or D_2O at pH or pD 7 after 48 h at 60 °C. **Σ Integral** is the total sum of integration in the (C2)–H and (C3)–H region of the ^{1}H NMR spectrum (3.5 – 4.5 ppm), using **MPA** and 2-carboxyethyldisulfide (the aerial oxidation product of **MPA**) as the internal standard, before (**t = 0**) and after heating at 60 °C for 48 h (**t = 48**). This value was further verified by the total integration of the $COCH_3$ region between 1.7 – 2.1 ppm of the ^{1}H NMR spectrum. The total integral intensity of the $COCH_3$ region after 48 h was completely recovered (>98%) in all cases. **Deuteration (%)** is the amount of (C2)–H and/or (C3)–H deuteration that was calculated by comparing the total ^{1}H NMR integral analysis of the (C2)–H and (C3)–H regions (3.5–4.5 ppm) before (**t = 0**) and after incubation of the reaction at 60 °C for 48 h (**t = 48**).

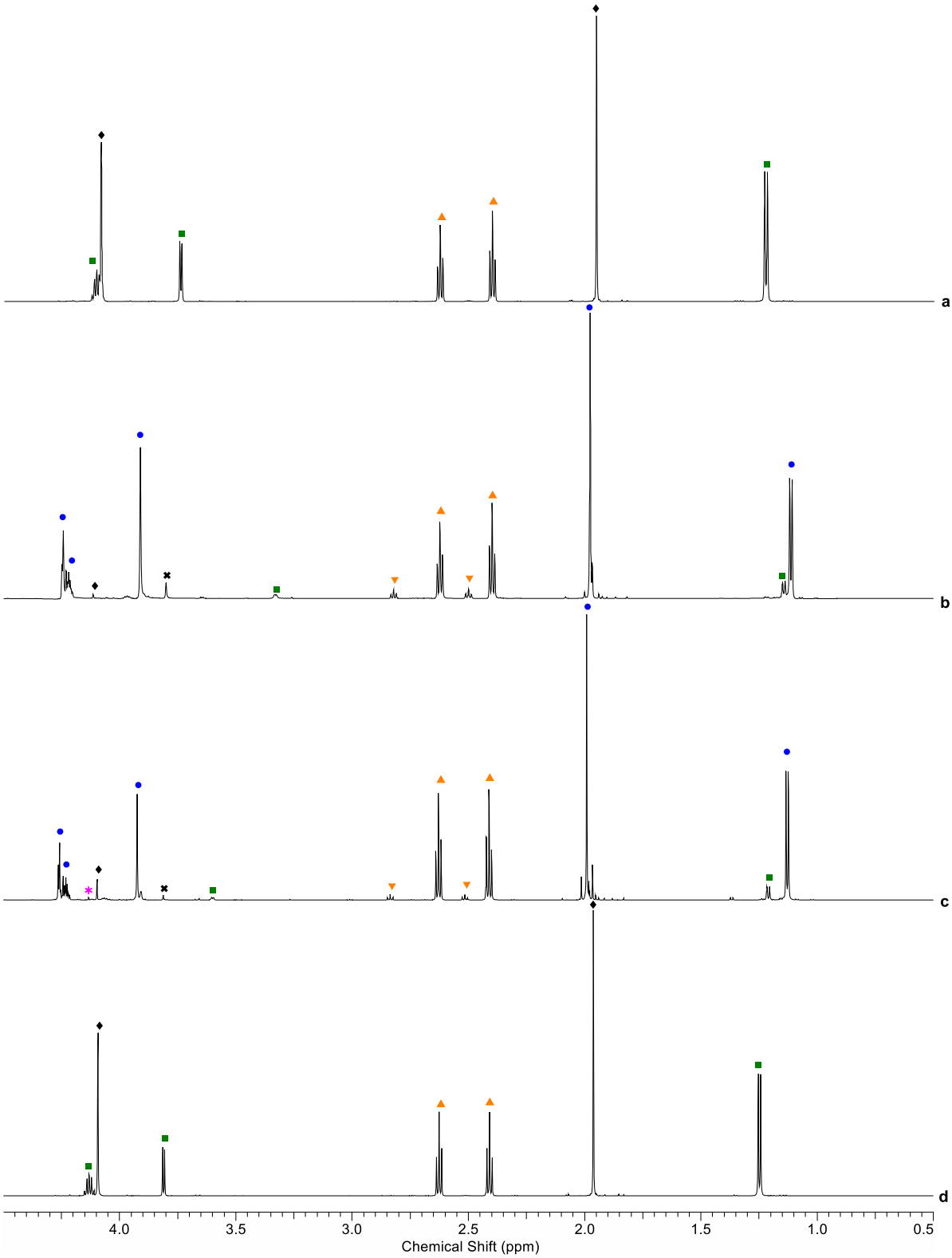


Fig. S199. ^{1}H NMR (600 MHz, noesygppr1d, 0.5–4.5 ppm) spectra to show the reaction of *N*-acetylglycine nitrile **Ac-Gly-CN** (200 mM) with L-threoninamide **Thr-NH$_2$** (200 mM) and 3-mercaptopropionic acid **MPA** (200 mM) after heating for 48 h at 60 °C in H_2O (pH 7) or D_2O (pD 7). a) **Ac-Gly-CN**, **Thr-NH$_2$** and **MPA** in H_2O at pH 7 before heating at 60 °C. b) **Ac-Gly-CN**, **Thr-NH$_2$** and **MPA** in H_2O at pH 7 after heating at 60 °C. c) **Ac-Gly-CN**, **Thr-NH$_2$** and **MPA** in D_2O at pD 7 after heating at 60 °C. d) **Ac-Gly-CN**, **Thr-NH$_2$** and **MPA** in D_2O at pD 7 before heating at 60 °C. ▼ = 2-Carboxyethyldisulfide (formed by aerial oxidation of **MPA** ▲). ✳ = *N*-(2-amino-2-iminoethyl)acetamide, **Ac-Gly^{N}-NH$_2$**. × = *N*-acetylglycinamide, **Ac-Gly-NH$_2$**.

Competition experiments

Competitive coupling of glycine and glycinamide with N-acetylglycine nitrile

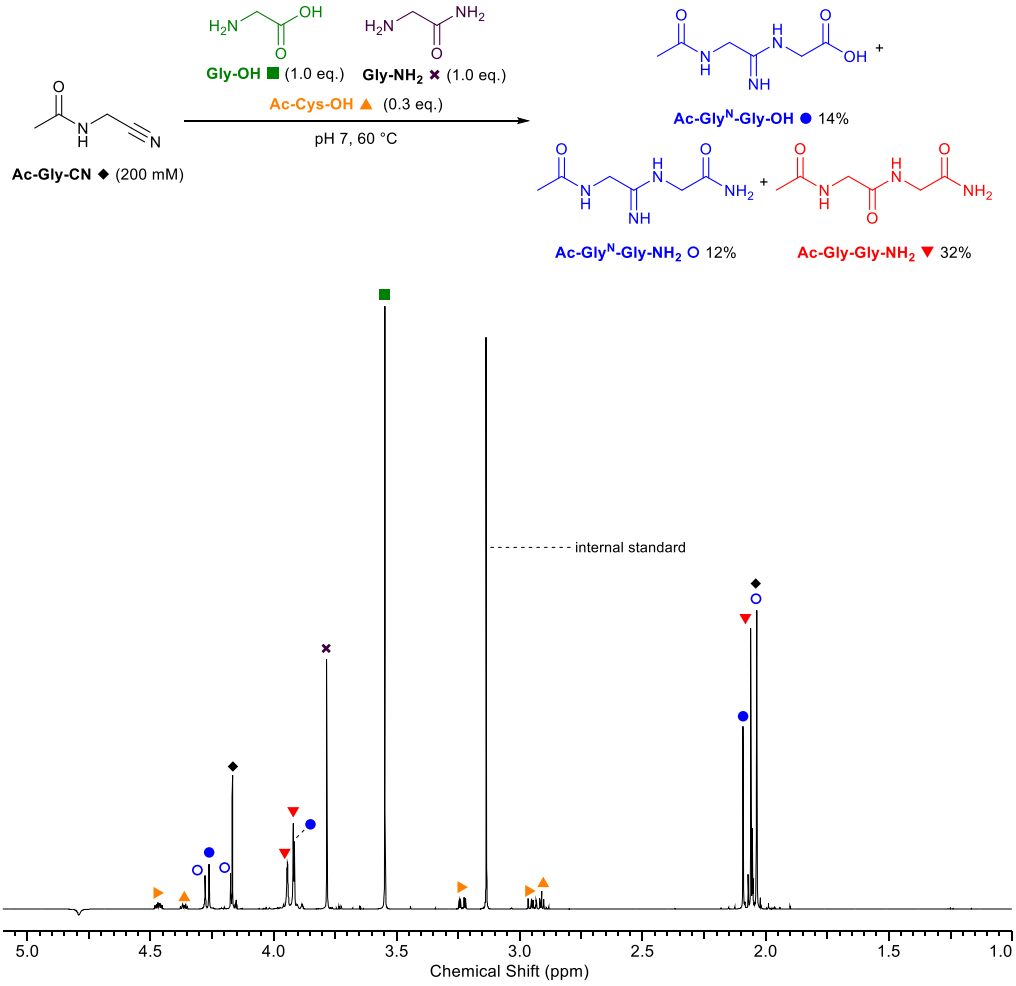


Fig. S200. ^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d, 1.0-5.0 ppm) spectrum to show the reaction of *N*-acetylglycine nitrile (**Ac-Gly-CN**, 200 mM) with glycine (**Gly-OH**, 200 mM), glycinamide (**Gly-NH$_2$**, 200 mM), and *N*-acetyl-L-cysteine (**Ac-Cys-OH**, 60 mM, 0.3 eq.) with MSM (50 mM) as the internal standard after 24 h at pH 7 and 60 °C. ▶ = *N*,*N*′-diacetyl-L-cystine (formed by aerial oxidation of **Ac-Cys-OH**).

^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d) *(2-acetamido-1-iminoethyl)glycine*, **Ac-Gly^{N}-Gly-OH** (●): δ_H 4.26 (2H, s, AcNHC*H*$_2$), 3.91 (2H, s, Gly-αH-COOH), 2.09 (3H, s, *H*$_3$C(CO)); *2-(2-acetamidoacetimidamido)acetamide*, **Ac-Gly^{N}-Gly-NH$_2$** (○): δ_H 4.28 (2H, s, AcNHC*H*$_2$), 4.17 (2H, s, Gly-αH-CONH$_2$), 2.03 (3H, s, *H*$_3$C(CO)); *N-Acetylglycylglycinamide*, **Ac-Gly-Gly-NH$_2$** (▼): δ_H 3.94 (2H, br. s., AcNHC*H*$_2$), 3.92 (2H, s, Gly-αH-CONH$_2$), 2.06 (3H, s, *H*$_3$C(CO)); *Glycine*, **Gly-OH** (■): δ_H 3.55 (2H, s, C*H*$_2$); *Glycinamide*, **Gly-NH$_2$** (✖): δ_H 3.78 (2H, s, C*H*$_2$).

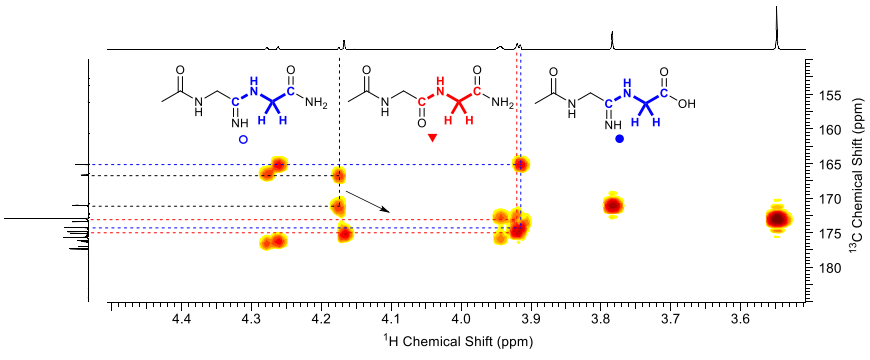
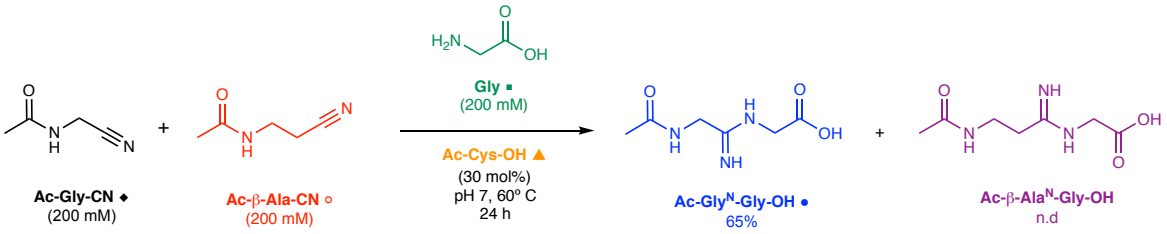


Fig. S201. ^{1}H–^{13}C HMBC (^{1}H: 700 MHz [3.50–4.50 ppm], ^{13}C: 176 MHz [150-185 ppm], H_2O/D_2O 9:1) spectrum showing the diagnostic $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling of **Gly**-αH-COOH in **Ac-Gly^{N}-Gly-OH** at 3.91 ppm with two resonances at 174 and 165 ppm, and **Gly**-αH-NH$_2$ in **Ac-Gly^{N}-Gly-NH$_2$** at 4.18 ppm with two resonances at 171 and 167 ppm and **Gly**-αH-NH$_2$ in **Ac-Gly-Gly-NH$_2$** at 3.92 ppm with two resonances at 175 and 173 ppm which is characteristic of amidine and amide bond formation of **Gly-OH** and **Gly-NH$_2$**. See Fig. S200 for expanded and labelled ^{1}H NMR spectrum.

Catalytic synthesis of proteinogenic α–peptides in the presence of non-proteinogenic substrates

Catalytic coupling of N-acetylglycine nitrile with glycine in the presence N-acetyl-β-alanine nitrile



N-Acetylglycine nitrile **Ac-Gly-CN** (200 mM), glycine **Gly** (200 mM), *N*-acetyl-β-alanine nitrile (200 mM) and *N*-acetyl-L-cysteine **Ac-Cys-OH** (60 mM), were adjusted to pH 7 with 1 – 4M HCl/NaOH and the solution was incubated at 60 °C for 24 h. The reaction was then analysed and quantified by NMR spectroscopy (^{1}H, ^{13}C, ^{1}H–^{13}C HMBC, ^{1}H–^{1}H COSY, ^{1}H–^{13}C HSQC). **Ac-Gly^{N}-Gly-OH** (65%) was observed, but no coupling of **Ac-β-Ala-CN** and **Gly** (to produce **Ac-β-Ala^{N}-Gly-OH**) could be detected (Fig. S202a). Additionally, a separate control experiment containing only **Ac-β-Ala-CN** (200 mM), **Gly** (200 mM) and **Ac-Cys-OH** (60 mM) at pH 7 and 60 °C gave no detectable **Ac-β-Ala^{N}-Gly-OH** after 24 h (Fig. S202b).

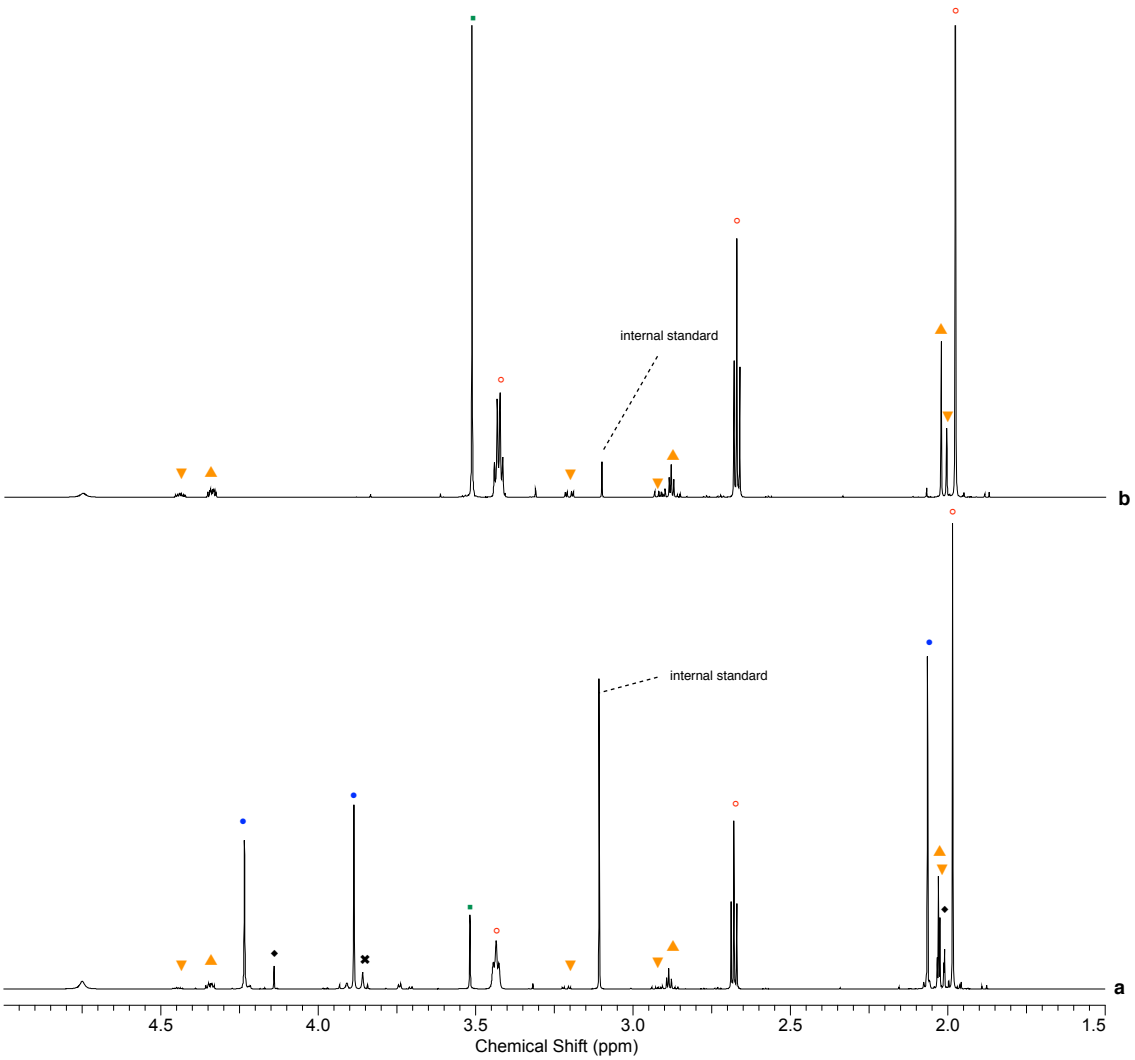
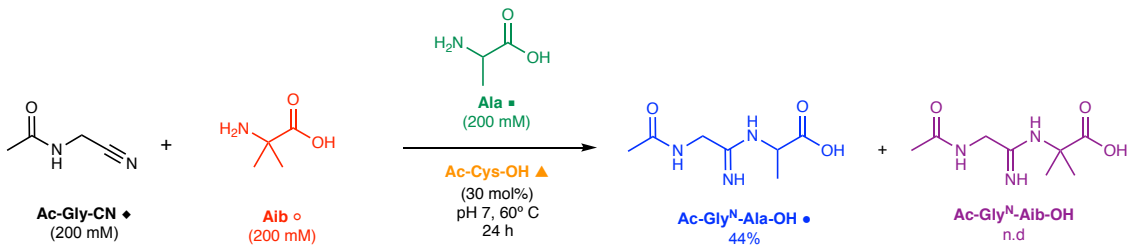


Fig. S202. ^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d, 1.5–50 ppm) spectrum to show **a**) the reaction of *N*-acetylglycine nitrile (**Ac-Gly-CN**, 200 mM), *N*-acetyl-β-alanine nitrile (**Ac-β-Ala-CN**, 200 mM), glycine (**Gly**, 200 mM) and *N*-acetyl-L-cysteine (**Ac-Cys-OH**, 60 mM, 0.3 eq.) after 24 h at pH 7 and 60 °C; **b**) the corresponding control reaction of *N*-acetyl-β-alanine nitrile (**Ac-β-Ala-CN**, 200 mM), glycine (**Gly**, 200 mM) and *N*-acetyl-L-cysteine (**Ac-Cys-OH**, 60 mM, 0.3 eq.) after 24 h at pH 7 and 60 °C, where no coupling of **Ac-β-Ala-CN** and **Gly** was observed. ▶ = *N,N'*-diacetyl-L-cystine (formed by aerial oxidation of **Ac-Cys-OH**), ✖ = *N*-acetylglycinamide **Ac-Gly-NH$_2$**.

Catalytic coupling of N-acetylglycine nitrile with DL-alanine in the presence of α-aminoisobutyric acid



N-Acetylglycine nitrile **Ac-Gly-CN** (200 mM), DL-alanine **Ala** (200 mM), α-aminoisobutyric acid (200 mM) and *N*-acetyl-L-cysteine **Ac-Cys-OH** (60 mM), were adjusted to pH 7 with 1 – 4M HCl/NaOH and the solution was incubated at 60 °C for 24 h. The reaction was then analysed and quantified by NMR spectroscopy (^{1}H, ^{13}C, ^{1}H–^{13}C HMBC, ^{1}H–^{1}H COSY, ^{1}H–^{13}C HSQC). **Ac-Gly^{N}-Ala-OH** (44%) was observed, but no coupling of **Aib** and **Ac-Gly-CN** (to produce **Ac-Gly^{N}-Aib-OH**) could be detected (Fig. S203a). Additionally, a separate control experiment containing only **Ac-Gly-CN** (200 mM), **Aib** (200 mM) and **Ac-Cys-OH** (60 mM) at pH 7 and 60 °C gave no detectable **Ac-Gly^{N}-Aib-OH** after 24 h (Fig. S203b).

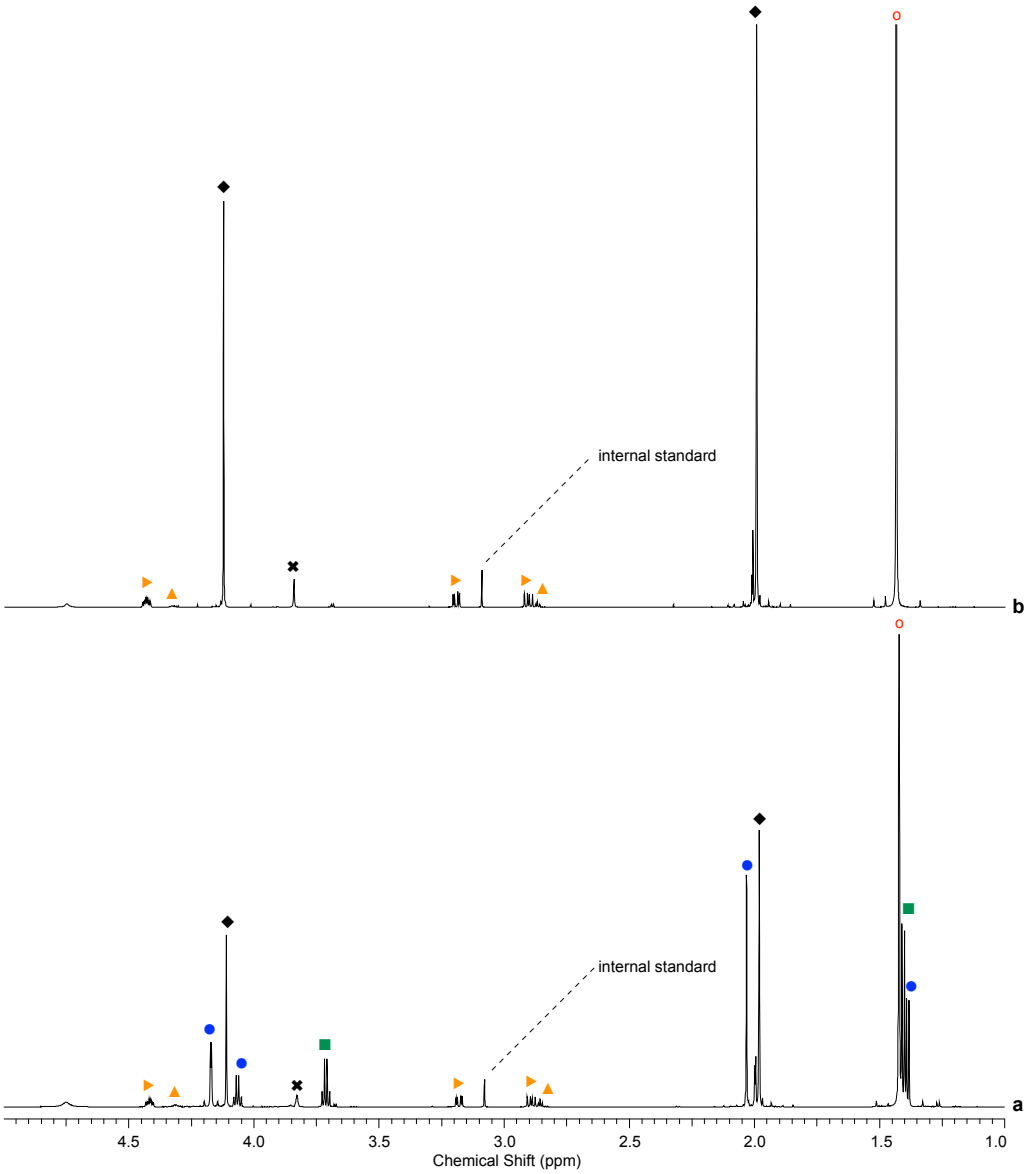
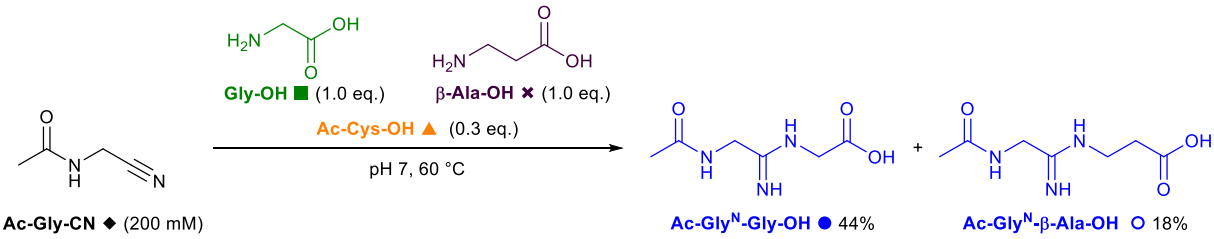


Fig. S203. ^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d, 1.5–5.0 ppm) spectrum to show **a**) the reaction of *N*-acetylglycine nitrile (**Ac-Gly-CN**, 200 mM), α-aminoisobutyric acid (**Aib**, 200 mM), DL-alanine (**Ala**, 200 mM) and *N*-acetyl-L-cysteine (**Ac-Cys-OH**, 60 mM, 0.3 equiv,) after 24 h at pH 7 and 60 °C; **b**) the corresponding control reaction of *N*-acetylglycine nitrile (**Ac-Gly-CN**, 200 mM), α-aminoisobutyric acid (**Aib**, 200 mM) and *N*-acetyl-L-cysteine (**Ac-Cys-OH**, 60 mM, 0.3 equiv.) after 24 h at pH 7 and 60 °C, where no coupling of **Ac-Gly-CN** and **Aib** was observed. ▶ = *N,N′*-diacetyl-L-cystine (formed by aerial oxidation of **Ac-Cys-OH**), ✖ = *N*-acetylglycinamide **Ac-Gly-NH$_2$**.

Catalytic coupling of N-acetylglycine nitrile with glycine in the presence of β-alanine



N-Acetylglycine nitrile **Ac-Gly-CN** (200 mM), glycine **Gly** (200 mM), β-alanine **β-Ala-OH** (200 mM) and *N*-acetyl-L-cysteine **Ac-Cys-OH** (60 mM), were adjusted to pH 7 with 1 – 4M HCl/NaOH and the solution was incubated at 60 °C for 24 h. The reaction was then analysed and quantified by NMR spectroscopy (^{1}H, ^{13}C, ^{1}H–^{13}C HMBC, ^{1}H–^{1}H COSY, ^{1}H–^{13}C HSQC). **Ac-Gly^{N}-Gly-OH** (44%) was observed alongside **Ac-Gly^{N}-β-Ala-OH** (18%) (Fig. S204b). Additionally, a separate control experiment containing only **Ac-Gly-CN** (200 mM), **β-Ala-OH** (200 mM) and **Ac-Cys-OH** (60 mM) at pH 7 and 60 °C gave **Ac-Gly^{N}-β-Ala-OH** (56%) after 24 h (Fig. S204a).

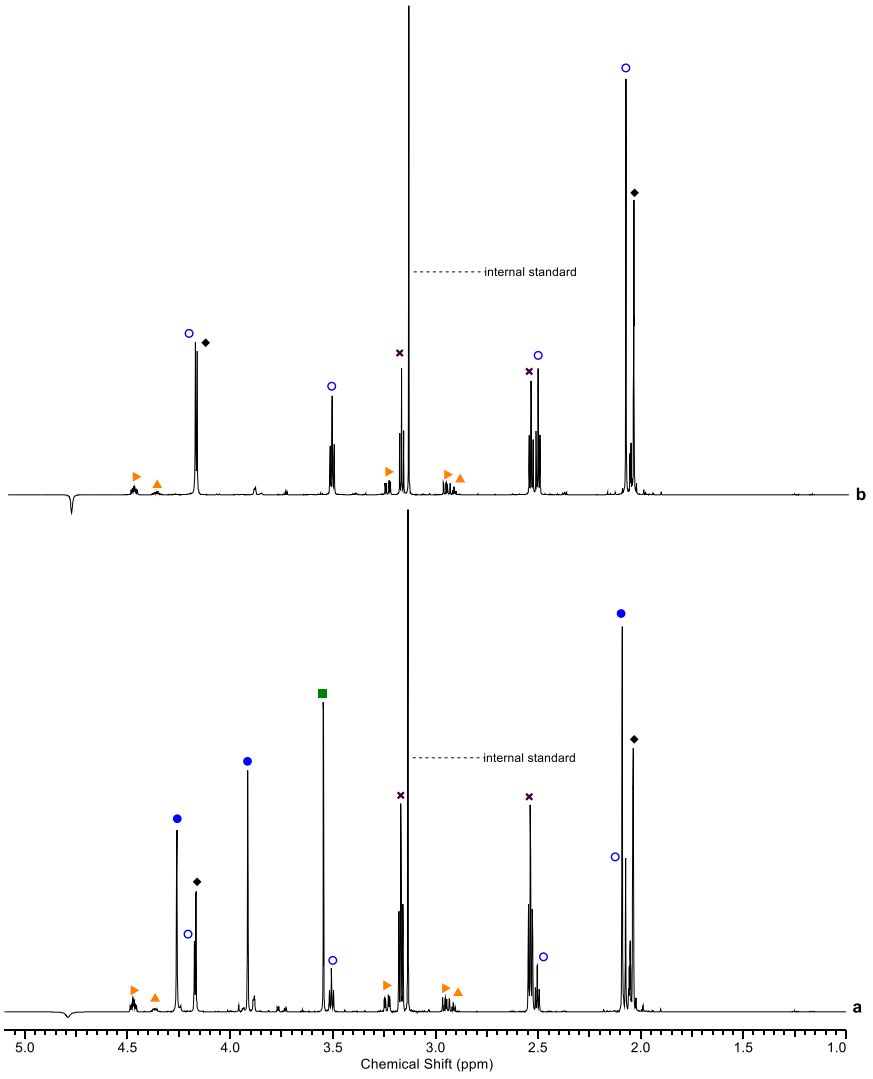


Fig. S204. ^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d, 1.0-5.0 ppm) spectrum to show **a**) the competitive ligation of *N*-acetylglycine nitrile (**Ac-Gly-CN**, 200 mM) with glycine (**Gly-OH**, 200 mM), β-alanine (**β-Ala-OH**, 200 mM), and *N*-acetyl-L-cysteine (**Ac-Cys-OH**, 60 mM, 0.3 eq.) with MSM (50 mM) as the internal standard after 24 h at pH 7 and 60 °C; **b**) the corresponding control reaction *N*-acetylglycine nitrile (**Ac-Gly-CN**, 200 mM) with β-alanine (**β-Ala-OH**, 200 mM) and *N*-acetyl-L-cysteine (**Ac-Cys-OH**, 60 mM, 0.3 eq.) with MSM (50 mM) as the internal standard after 24 h at pH 7 and 60 °C. ► = *N,N'*-diacetyl-L-cystine (formed by aerial oxidation of **Ac-Cys-OH**).

^{1}H NMR (700 MHz, H_2O/D_2O 9:1, noesygppr1d) *(2-acetamido-1-iminoethyl)glycine*, **Ac-Gly^{N}-Gly-OH** (●): δ_H 4.26 (2H, s, AcNHCH_2), 3.91 (2H, s, Gly-αH-COOH), 2.09 (3H, s, H_3C(CO)); *3-(2-acetamidoacetimidamido)propanoic acid*, **Ac-Gly^{N}-β-Ala-OH** (○): δ_H 4.18 (2H, s, AcNHCH_2), 3.51 (2H, t, *J* = 6.6 Hz, CH_2CH$_2$COOH), 2.50 (2H, t, *J* = 6.6 Hz, CH$_2$CH_2COOH) 2.07 (3H, s, H_3C(CO)); *Glycine*, **Gly-OH** (■): δ_H 3.55 (2H, s, CH_2); *β-Alanine*, **β-Ala-OH** (✖): δ_H 3.17 (2H, t, *J* = 6.6 Hz, CH_2CH$_2$COOH), 2.54 (2H, t, *J* = 6.6 Hz, CH$_2$CH_2COOH).

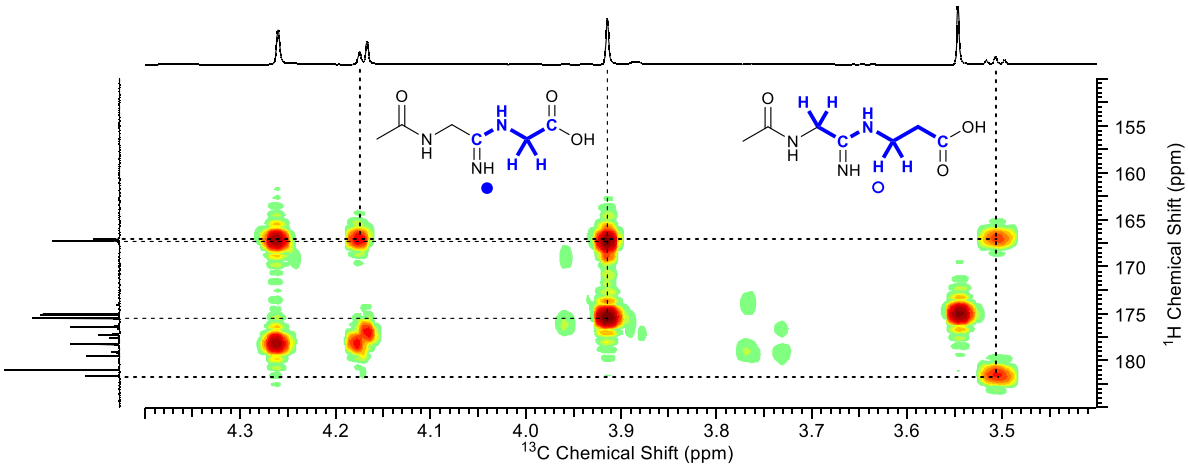
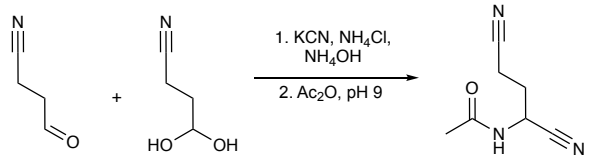


Fig. S205. ^{1}H–^{13}C HMBC (^{1}H: 700 MHz [3.50–4.50 ppm], ^{13}C: 176 MHz [150-185 ppm], H_2O/D_2O 9:1) spectrum showing the diagnostic $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling of **Gly**-αH-COOH in **Ac-Gly^{N}-Gly-OH** at 3.91 ppm with two resonances at 175 and 167 ppm, and **Ac-Gly**-αH at 4.17 ppm and **β-Ala**-αH-OH at 3.51 ppm in **Ac-Gly^{N}-β-Ala-OH** with a resonance at 167 ppm, which is characteristic of amidine bond formation of **Gly** and **β-Ala-OH**, respectively . See Fig. S204 for expanded and labelled ^{1}H NMR spectrum.

Miscellaneous preparative syntheses

N-Acetyl-2-aminoglutaronitrile (N-acetylglutamine dinitrile)



Ac-Glx-CN

Ammonium hydroxide (25%; 9 mL), ammonium chloride (828 mg, 15.4 mmol) and potassium cyanide (604 mg, 9.2 mmol) were added to a mixture of 3-cyanopropionaldehyde and 4,4-dihydroxybutanenitrile. The mixture was then stirred for 72 h at room temperature. The reaction was partially concentrated *in vacuo* to remove excess ammonia. Acetic anhydride (2 mL, 21.1 mmol) was then added to the reaction mixture, the solution was adjusted to pH 9.0, and stirred for 12 h at room temperature. The reaction mixture was concentrated to dryness *in vacuo* and the crude residue was purified by flash column chromatography (SiO_2; eluting with ethyl acetate) to give *N*-acetyl-2-aminoglutaronitrile (*N*-acetylglutamine dinitrile) **Ac-Glx-CN** (702 mg, 4.6 mmol, 60%) as a yellow oil. ^{1}H NMR (700 MHz, H_2O, noesygppr1d): δ 4.94 (1H, dd, *J* = 8.9, 6.0 Hz, C*H*(CH_2CH_2CN)), 2.74–2.67 (2H, m, CH(C*H*$_2$$CH_2CN$)), 2.37–2.27 (2H, m, CH(CH_2C*H*$_2$CN)), 2.08 (3H, s, *H*$_3$C(CO)-). ^{13}C NMR (176 MHz; H_2O): δ 174.1, 119.7, 117.9, 39.7, 27.1, 21.6, 13.2. HRMS-ESI $[M+H]^+$ calculated for formula $C_7H_{10}N_3O^+$, 152.0824; found 152.0826.

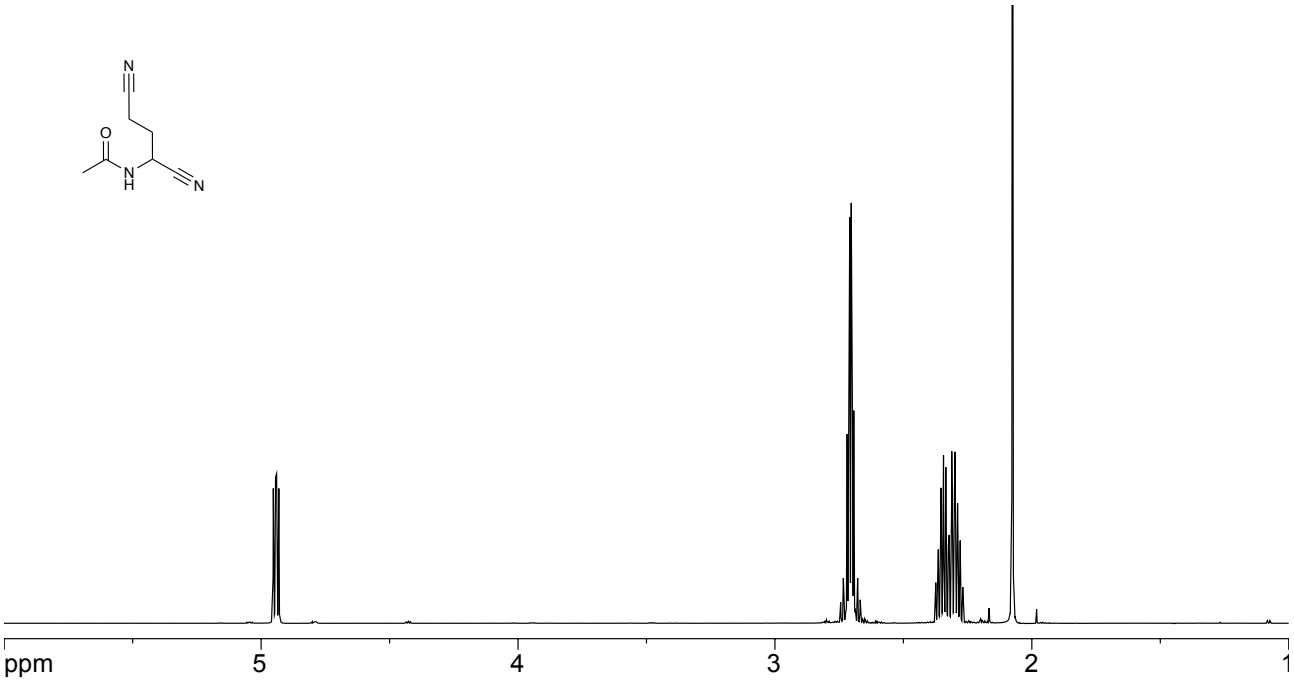


Fig. S206. ^{1}H NMR (700 MHz, H_2O, noesygppr1d, 1.00–6.00 ppm) spectrum of *N*-acetyl-2-aminoglutaronitrile **Ac-Glx-CN**.

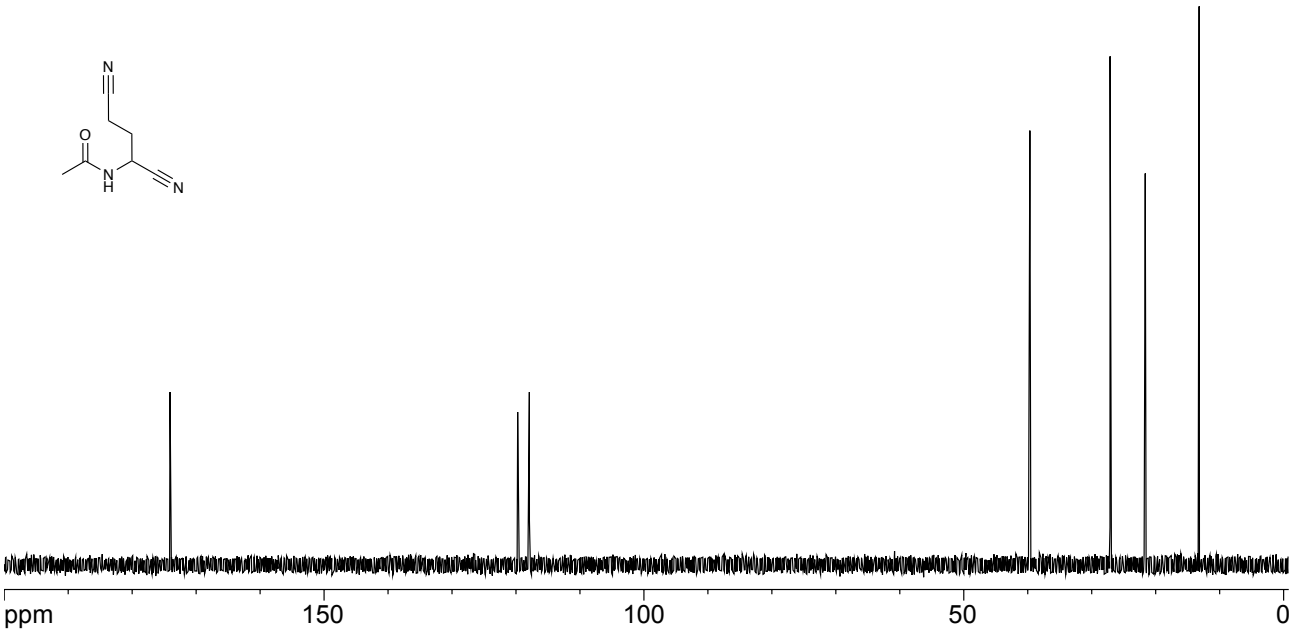
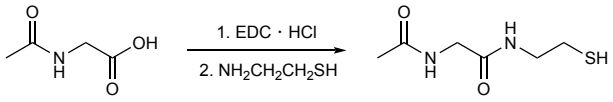


Fig. S207. ^{13}C NMR (176 MHz, H_2O, 0–200 ppm) spectrum of *N*-acetyl-2-aminoglutaronitrile **Ac-Glx-CN**.

N-Acetylglycylcysteamine



1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (**EDC**·HCl; 1.29 g, 7.50 mmol) and *N*-acetylglycine (586 mg, 5.00 mmol) were stirred in chloroform (20 mL) for 30 min at room temperature. Cysteamine (463 mg, 6.00 mmol) was added in one portion and stirring was continued under a nitrogen atmosphere for 24 h. The solvent was then removed *in vacuo*, and the residue purified by flash column chromatography (SiO_2; 0:100 →15:85; MeOH:CH_2Cl_2) to give *N*-acetylglycylcysteamine as a white solid (360 mg, 41%); m.p. 149-150 °C (MeOH:CH_2Cl_2); R_f = 0.6 (10:90 MeOH:CH_2Cl_2). ^{1}H NMR (600 MHz, $CDCl_3$) 6.91 (1H, s br., NH), 6.61 (1H, s br., NH), 3.94 (2H, app.d, *J* = 5.2 Hz,Gly-CH_2), 3.46 (2H, app. q, *J* = 6.5 Hz, C*H*$_2$CH$_2$SH), 2.67 (2H, dt, *J* = 8.5, 6.5 Hz, CH$_2$C*H*$_2$SH), 2.05 (3H, s, $COCH_3$), 1.44 (1H, t, *J* = 8.5 Hz, CH$_2$S*H*). ^{13}C NMR (151 MHz, $CDCl_3$) 171.0 (**C**OCH_3), 169.22 (Gly-(C1)), 43.6 (Gly-(C2)), 42.6 (**C**H$_2$CH$_2$SH), 24.5 (CH$_2$**C**H$_2$SH), 23.1 (CO**C**H$_3$); HRMS (CI) found 177.061 $[M]^{+\cdot}$ mass for $C_6H_{12}O_2N_2$, requires 177.0692. IR (cm^{-1}) 3253 , 3072, 2957, 1672, 1560.

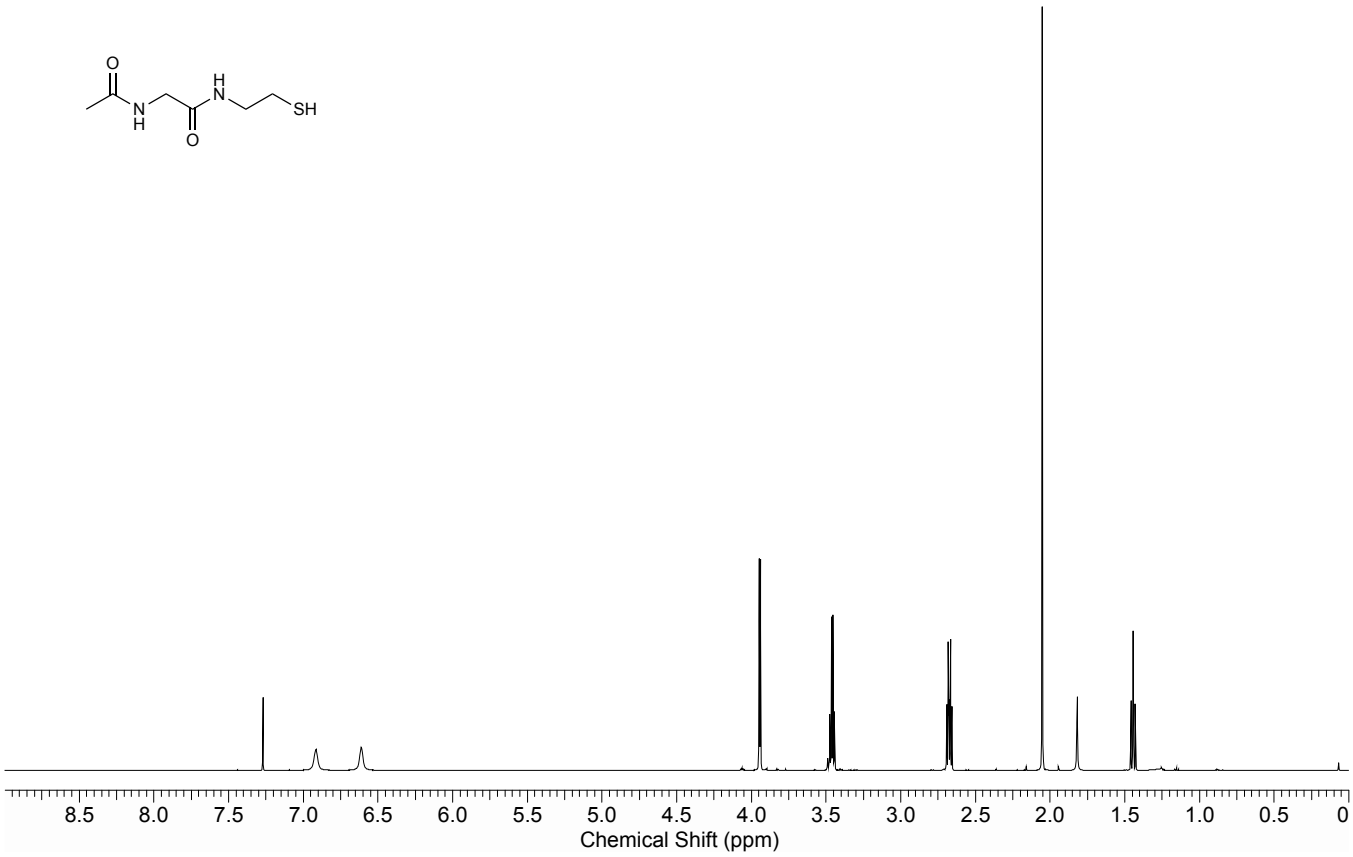


Fig. S208. ^{1}H NMR (600 MHz, $CDCl_3$, 0.0–9.0 ppm) spectrum to show *N*-acetylglycylcysteamine.

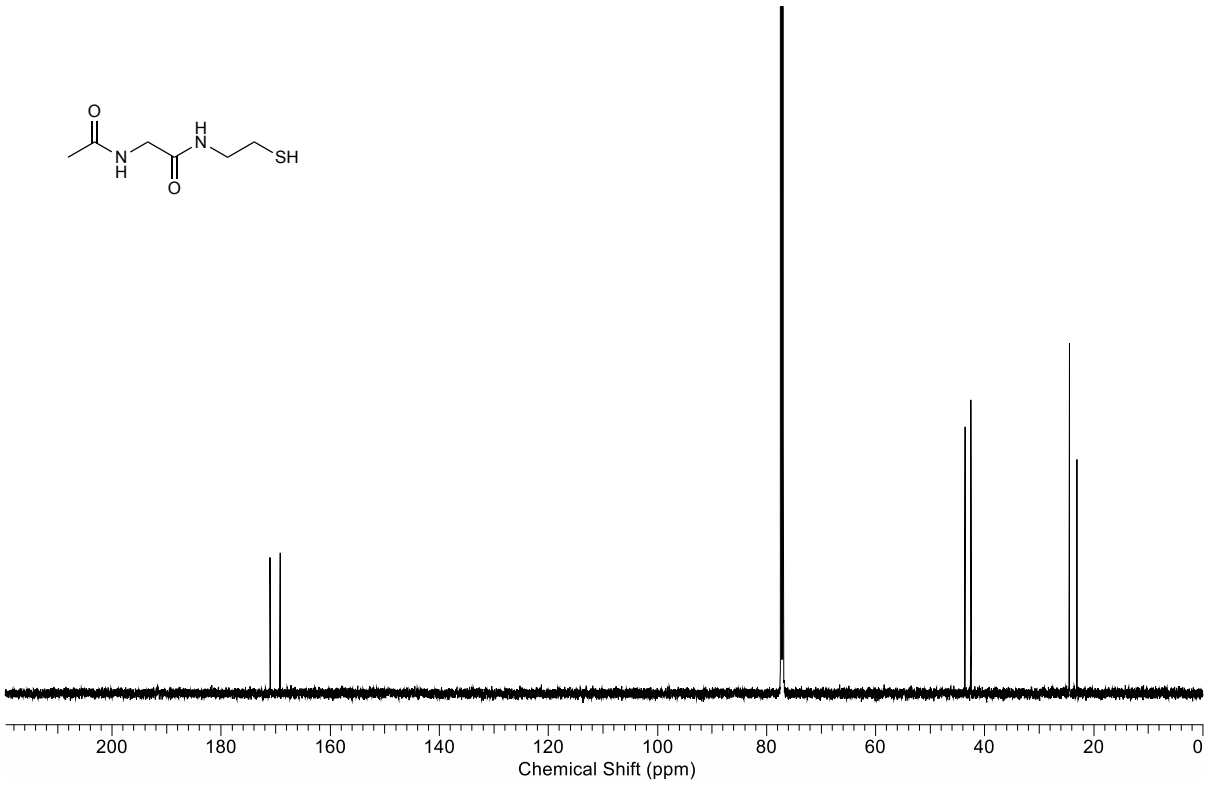


Fig. S209. ^{1}H NMR (151 MHz, $CDCl_3$, 0–220 ppm) spectrum to show *N*-acetylglycylcysteamine.

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