Supporting Information

pH-dependent peptide bond formation by the selective coupling of

α -amino acids in water

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Materials and methods. Reagents and solvents were obtained from Acros Organics, Alfa Aesar, Santa Cruz Biotechnology, Sigma-Aldrich, SYNTHON Chemicals GmbH & Co. KG and VWR International, and were used without further purification unless otherwise stated. A MettlerToledo SevenEasy pH Meter S20 combined with a ThermoFisher Scientific Orion 8103BN Ross semi-micro pH electrode was used to measure and adjust the pH to the desired value. ¹H-, and ¹³C-nuclear magnetic resonance (NMR) spectra were acquired using a Bruker Ultrashield 400 Plus or Bruker Ascend 400 operating at 400.13, and 100.62 MHz, respectively. Samples consisting of H₂O/D₂O mixtures were analysed using HOD suppression to collect ¹H-NMR spectroscopy data. Coupling constants (J) are given in Hertz (Hz) and the notations s, d, t, q, m, and b represent the multiplicities of singlet, doublet, triplet, quatruplet, multiplet, and broad signals, respectively. The yields of conversion were determined by relative integration of the signals in the ¹H-NMR spectra. Data analysis was performed using MestReNova (version 7.0). CAUTION! Methyl isonitrile is a volatile liquid at room temperature and it gives a very unpleasant smell. Solutions containing methyl isonitrile should better be handled in a well ventilated fume hood equipped with quenching solution, such as 0.1 M H₂SO₄ solution.

General procedure for the methyl isonitrile 1 mediated formation of amide bonds in water.

An aqueous solution (0.5 mL, H₂O/D₂O, 4:1) of Ac-Ala **2** (100 mM) and amine nucleophile **3** (50 mM) was adjusted to pH 3, 4 or 5 with HCl (1 M) and NaOH (1 M) solutions. Methyl isonitrile **1** (6 μ L, concentration in theory would be 200 mM, ~ 130 mM in fact, due to its volatility, based on ¹H-NMR relative integration) was then added, and the reaction was incubated for 6 hours at pH 3, 48 hours at pH 4 and 15 days at pH 5, respectively, at 23 °C. Then reactions were analyzed by ¹H-NMR spectroscopy at 23 °C. The pH had changed only slightly (< 0.2) by the time the isonitrile was totally consumed. The only exception was the reaction when glycine nitrile **3d** was used as amine nucleophile at pH 5. For this reaction, the pH was adjusted to 5 using HCl (1 M) once a day, or the pH increased to around 7 after 15 days.

Procedures for the formation of 9 and control experiments

An aqueous solution (0.5 mL, H₂O/D₂O, 4:1) of Ac-Ala **2** (100 mM) and glycolic acid **8** (50 mM) was adjusted to pH 3 or 4 with HCl (1 M) and NaOH (1 M) solutions. Methyl isonitrile **1** (6 μ L, concentration in theory would be 200 mM, ~ 130 mM in fact, due to its volatility, based on ¹H-NMR relative integration) was then added, and the reaction was incubated for 6 hours at pH 3 and 48 hours at pH 4, respectively, at 23 °C. For the reaction run at pH 4, the pH was maintained at 4 by using HCl (1 M) once a day. Then reactions were analyzed by ¹H-NMR spectroscopy at 23 °C. Control experiment by using methyl glycolate **11** instead of glycolic acid **8** were also run at pH 4.

Procedures for the competing experiment in a "one-pot" reaction

An aqueous solution (0.5 mL, H₂O/D₂O, 4:1) of Ac-Ala 2 (100 mM), glycine **3a** (25 mM), β -alanine **3b** (25 mM), glycylglycine **3c** (25 mM), glycineamide **3d** (25 mM), glycinenitrile **3e** (25 mM), methylammonium chloride **3f** (25 mM) and ammonium chloride **3g** (25 mM) was adjusted to pH 3 with HCl (1 M) and NaOH (1 M) solutions. Methyl isonitrile **1** (6 µL, concentration in theory would be 200 mM, ~ 130 mM in fact, due to its volatility, based on ¹H-NMR relative integration) was then added, and the reaction was incubated for 6 hours at pH 3 or 48 hours at pH 4, respectively, at 23 °C. Then reactions were analyzed by ¹H-NMR spectroscopy at 23 °C. The pH had changed only slightly (< 0.2) by the time the isonitrile was totally consumed.

Procedures for multiple rounds of peptide elongation mediated by methyl isonitrile

An aqueous solution (0.5 mL, H₂O/D₂O, 4:1) of Ac-Ala **2** (20 mM), glycine **3a** (100 mM) was adjusted to pH 3 or 4 with HCl (1 M) and NaOH (1 M) solutions. Methyl isonitrile **1** (5 μ L, ~ 110 mM in fact, due to its volatility, based on ¹H-NMR relative integration) was then added, and the reaction was incubated for 6 hours at pH 3 or 48 hours at pH 4, respectively, at 23 °C. Then reactions were analyzed by ¹H-NMR spectroscopy at 23 °C. The pH had changed only slightly (< 0.2) by the time the isonitrile was totally consumed. Further, the pH of the solution was adjusted to 5 and

analyzed again by ¹H-NMR to have better resolution of peaks in ¹H-NMR for quantification of products. Similar reaction was incubated for 15 days at pH 5, but the pH of the reaction was maintained at 5 using HCl (1 M) once a day.

Chemical synthesis of Ac-Ala-Gly-Gly-OMe

Ac-Ala **2** (100 mg, 0.76 mmol) and Glycylglycine methyl ester hydrochloride (279 mg, 1.5 mmol) were dissolved in 5 mL water. The pH of the mixture was adjusted to 6 followed by adding EDC·HCl (290 mg, 1.5 mmol). The reaction was incubated at 25 °C and the pH maintained below 6 with HCl (1 M) solution. After 2 hours at room temperature, 50 µL of the reaction mixture was mixed with 450 µL of water (with 20% D₂O) and checked by ¹H-NMR spectroscopy. After all the Ac-Ala was converted, the pH of reaction mixture was adjusted to 5. Then the mixture was passed thought a H⁺-formed Dowex-50 column, and washed with 50 mL of water. The combined solution was lyophilized to give the desired product as white powder (140 mg). ¹H NMR (400 MHz, 20 % D₂O, pH = 1) δ = 8.40 (t, *J* = 6.1 Hz), 8.27 (b), 8.13 (t), 4.21 (dq, *J* = 8.9, 7.0 Hz, 1H), 3.97 (d, *J* = 5.9 Hz, 2H), 3.89 (dd, *J* = 6.0, 2.3 Hz, 2H), 3.68 (s, 3H), 1.95 (s, 3H), 1.31 (d, *J* = 7.2 Hz, 3H); ¹³C NMR (101 MHz, 20 % D₂O, pH = 1) δ = 176.0, 174.5, 172.0, 172.0, 52.9, 50.1, 42.5, 41.2, 21.7, 16.5.

Chemical synthesis of Ac-Ala-β-Ala 4b

Ac-Ala (100 mg, 0.75 mmol) and β-Alanine methyl ester hydrochloride (210 mg, 1.5 mmol) were dissolved in 5 mL water. The pH of the mixture was adjusted to 6 followed by adding EDC·HCl (292 mg, 1.5 mmol). The reaction was incubated at 25 °C and the pH maintained below 6 with HCl (1 M) solution. After 2 hours at room temperature, 50 µL of the reaction mixture was mixed with 450 µL of water (with 20% D₂O) and checked by ¹H-NMR spectroscopy. After all the Ac-Ala was converted, the pH of the reaction mixture was adjusted to 5. Then the mixture was passed thought a H⁺-formed Dowex-50 column, and washed with 50 mL of water. The combined solution was lyophilized to give a light yellow powder (180 mg). 20 mg of the white powder (Ac-Ala-β-Ala-OMe) was dissolved in 0.5 mL water (with 20 % D₂O), and the solution was adjusted to pH 12. The mixed was monitored by ¹H-NMR spectroscopy at 23 °C until all the methyl ester was hydrolyzed. Then the solution was lyophilized again and re-dissolved in 0.5 mL water (20 % D₂O), the

solution was adjusted to pH 5 and monitored by NMR spectroscopy at 23 °C. ¹H NMR (400 MHz, 20 % D₂O, pH = 5) δ 8.17 (d, *J* = 6.0 Hz, 1H), 7.93 (d, *J* = 6.2 Hz, 1H), 4.21 – 4.09 (m, 1H), 3.34 (qd, *J* = 6.8, 2.8 Hz, 2H), 2.35 (t, *J* = 6.9 Hz, 2H), 1.96 (s, 3H), 1.28 (d, *J* = 7.2 Hz, 3H); ¹³C NMR (101 MHz, 20% D₂O, pH = 5) δ 179.6, 175.1, 174.2, 50.1, 36.5, 36.2, 21.8, 16.9.

Chemical synthesis of standard Ac-Ala-Gly-Gly 4c

15 mg of the white powder Ac-Ala-Gly-Gly-OMe was dissolved in 0.5 mL water (with 20 % D₂O), and the solution was adjusted to pH 12. The mixed was monitored by ¹H-NMR spectroscopy 23 °C until all the methyl ester was hydrolyzed. Then the solution was lyophilized again and re-dissolved in 0.5 mL water (20 % D₂O), the solution was adjusted to pH 5 and monitored by NMR spectroscopy at 23 °C. ¹H-NMR (400 Hz, 20 % D₂O, pH = 5) δ = 8.41 (t, *J* = 5.9 Hz), 8.29 (d, *J* = 5.7 Hz), 7.88 (b), 4.26 (dq, *J* = 9.3, 6.8 Hz, 1H), 3.93 – 3.89 (m, 2H), 3.73 (d, 2H), 1.98 (s, 3H), 1.34 (d, *J* = 7.2 Hz, 3H); ¹³C-NMR (101 Hz, 20 % D₂O, pH = 5) δ = 196.4, 176.0, 174.5, 171.2, 50.1, 43.3, 42.6, 21.8, 16.6.

Chemical synthesis of standard Ac-Ala-Gly-NH₂ 4d

Ac-Ala (100 mg, 0.75 mmol) and glycinamide (169 mg, 1.5 mmol) were dissolved in 5 mL water. The pH of the mixture was adjusted to 6 followed by adding EDC·HCl (292 mg, 1.5 mmol). The reaction was incubated at 25 °C and the pH maintained below 6 with HCl (1 M) solution. After 2 hours at room temperature, 50 μ L of the reaction mixture was mixed with 450 μ L of water (with 20% D₂O) and checked by ¹H-NMR spectroscopy. After all the Ac-Ala was converted, the pH of the reaction mixture was adjusted to 5. Then the mixture was passed thought a H⁺-formed Dowex-50 column, and washed with 50 mL of water. The combined solution was lyophilized to give a white powder. 20 mg of the white powder was dissolved in 20 % D₂O/H₂O and the solution was monitored by NMR spectroscopy. ¹H NMR (400 MHz, 20 % D₂O) δ 4.22 (q, *J* = 7.2 Hz, 1H), 3.92 – 3.76 (m, 2H), 1.98 (s, 3H), 1.33 (d, *J* = 7.2, 3H); ¹³C NMR (101 MHz, 20 % D₂O) δ 176.1, 174.5, 174.4, 50.2, 42.3, 21.7, 16.4.

Chemical synthesis of standard Ac-Ala-Gly-CN 4e

Ac-Ala (100 mg, 0.75 mmol) and glycine nitrile hydrochloride (141 mg, 1.5 mmol) were dissolved in 5 mL water. The pH of the mixture was adjusted to 6 followed by

adding EDC·HCl (292 mg, 1.5 mmol). The reaction was incubated at 25 °C and the pH maintained below 6 with HCl (1 M) solution. After 2 hours at room temperature, 50 µL of the reaction mixture was mixed with 450 µL of water (with 20% D₂O) and checked by ¹H-NMR spectroscopy. After all the Ac-Ala was converted, the pH of the reaction mixture was adjusted to 5. Then the mixture was passed thought a H⁺-formed Dowex-50 column, and washed with 50 mL of water. The combined solution was lyophilized to give a light yellow powder. 20 mg of the light yellow powder was dissolved in 20 % D₂O/H₂O and the solution was monitored by NMR spectroscopy. ¹H NMR (400 MHz, 20 % D₂O) δ 4.22 (q, *J* = 7.3 Hz, 1H), 4.14 (s, 2H), 1.97 (s, 3H), 1.31 (d, *J* = 7.3 Hz, 3H); ¹³C NMR (101 MHz, 20 % D₂O) δ 175.9, 174.3, 116.9, 49.8, 27.7, 21.7, 16.4.

Chemical synthesis of standard Ac-Ala-NHCH₃ 4f

Ac-Ala (100 mg, 0.75 mmol) and methylammonium chloride (103 mg, 1.5 mmol) were dissolved in 5 mL water. The pH of the mixture was adjusted to 6 followed by adding EDC·HCl (292 mg, 1.5 mmol). The reaction was incubated at 25 °C and the pH maintained below 6 with HCl (1 M) solution. After 4 hours at room temperature, 50 μ L of the reaction mixture was mixed with 450 μ L of water (with 20% D₂O) and checked by ¹H-NMR. After all the Ac-Ala was converted, the pH of the reaction mixture was adjusted to 5. Then the mixture was passed thought a H⁺-formed Dowex-50 column, and washed with 50 mL of water. The combined solution was lyophilized to give a white powder. 20 mg of the white powder was dissolved in 20 % D₂O/H₂O and the solution was monitored by NMR spectroscopy. ¹H NMR (400 MHz, 20 % D₂O) δ 4.19 - 4.11 (m, 1H), 2.67 (d, J = 4.7 Hz, 3H), 1.96 (s, 3H), 1.28 (d, J = 7.3 Hz, 3H); ¹³C NMR (101 MHz, 20 % D₂O) δ 175.8, 174.2, 50.0, 26.0, 21.8, 16.7.

Chemical synthesis of standard Ac-Ala-NH₂ 4g

Ac-Ala (100 mg, 0.75 mmol) and ammonium chloride (82 mg, 1.5 mmol) were dissolved in 5 mL water. The pH of the mixture was adjusted to 7 followed by adding EDC·HCl (292 mg, 1.5 mmol). The reaction was incubated at 25 °C and the pH maintained below 7 with HCl (1 M) solution. After 4 hours at room temperature, 50 μ L of the reaction mixture was mixed with 450 μ L of water (with 20% D₂O) and checked by ¹H-NMR spectroscopy. After all the Ac-Ala was converted, the pH of the reaction mixture was adjusted to 5. Then the mixture was passed thought a H⁺-formed

Dowex-50 column, and washed with 50 mL of water. The combined solution was lyophilized to give a white powder. 20 mg of the white powder was dissolved in 20 % D₂O/H₂O and the solution was monitored by NMR spectroscopy. ¹H NMR (400 MHz, 20 % D₂O) δ 4.19 (qd, *J* = 7.2, 6.0 Hz, 1H), 1.96 (s, 3H), 1.32 (d, *J* = 7.3 Hz, 3H); ¹³C NMR (101 MHz, 20 % D₂O) δ 178.3, 174.2, 49.7, 21.8, 16.8.

Chemical synthesis of standard Ac-Ala-Gly-Gly-Gly 4h

Ac-Ala-Gly¹ (190 mg, 1 mmol) and Glycylglycine methyl ester hydrochloride (370 mg, 2 mmol) were dissolved in 5 mL water. The pH of the mixture was adjusted to 6 followed by adding EDC·HCl (386 mg, 2 mmol). The reaction was incubated at 25 °C and the pH maintained below 6 with HCl (1 M) solution. After 2 hours at room temperature, 50 µL of the reaction mixture was mixed with 450 µL of water (with 20% D₂O) and checked by ¹H-NMR spectroscopy. After all the Ac-Ala-Gly was converted, the pH of the reaction mixture was adjusted to 5. Then the mixture was passed thought a H⁺-formed Dowex-50 column, and washed with 50 mL of water. The combined solution was lyophilized to give a white powder (180 mg). 20 mg of the white powder (Ac-Ala-Gly-Gly-OMe) was dissolved in 0.5 mL water (with 20 % D_2O), and the solution was adjusted to pH 12. The mixed was monitored by ¹H-NMR spectroscopy at 23 °C until all the methyl ester was hydrolyzed. Then the solution was lyophilized again and re-dissolved in 0.5 mL water (20 % D₂O), the solution was adjusted to pH 5 and monitored by NMR spectroscopy at 23 °C. ¹H NMR (400 MHz, 20 % D_2O , pH = 5) δ 8.47 (t, J = 5.7 Hz), 8.31 (d, J = 5.8 Hz), 8.23 (t, J = 6.2 Hz), 7.97 (t, J = 5.9 Hz), 4.28 (dq, J = 7.1, 4.6 Hz, 1H), 4.02 - 3.89 (m, 4H), 3.77 - 3.72 (d, 2H), 2.01 (s, 3H), 1.36 (d, J = 7.2 Hz, 3H); ¹³C (101 MHz, 20 % D_2O , pH = 5) δ 176.5, 176.2, 174.5, 172.2, 171.2, 50.1, 43.3, 42.8, 42.6, 21.9, 16.6.

Chemical synthesis of standard Ac-Ala-Gly-Gly-Gly-Gly 4i

Ac-Ala-Gly-Gly-OMe (120 mg, 0.4 mmol) was dissolved in 4 mL water, and the solution was adjusted to pH 12. After 2 hours at room temperature, 50 uL of the reaction mixture was mixed with 450 uL of water (with 20% D₂O) and checked by ¹H-NMR spectroscopy. Once all the methyl ester was hydrolyzed, the pH of the mixture was adjusted to 5. Then glycylglycine methyl ester hydrochloride (148 mg, 0.8 mmol) was added to the aqueous solution. The pH of the mixture was adjusted to 6 followed by adding EDC·HCl (155 mg, 0.8 mmol). The reaction was incubated at

25 °C and the pH maintained below 6 with HCl (1 M) solution. After 2 hours at room temperature, 50 µL of the reaction mixture was mixed with 450 µL of water (with 20% D₂O) and checked by ¹H-NMR spectroscopy. After all the Ac-Ala-Gly was converted, the pH of reaction mixture was adjusted to 5. Then the mixture was passed thought a H⁺-formed Dowex-50 column, and washed with 50 mL of water. The combined solution was lyophilized to give a white powder (120 mg). 20 mg of the white powder (Ac-Ala-Gly-Gly-Gly-OMe) was dissolved in 0.5 mL water (with 20 % D₂O), and the solution was adjusted to pH 12. The mixed was monitored by ¹H-NMR spectroscopy at 23 °C until all the methyl ester was hydrolyzed. Then the solution was adjusted to pH 5 and monitored by NMR spectroscopy at 23 °C. ¹H NMR (400 MHz, 20 % D₂O, pH = 5) δ 8.46 (t, *J* = 5.9 Hz), 8.30-8.28 (m), 7.95 (t, J = 5.9 Hz), 4.29 - 4.17 (m, 1H), 3.99 - 3.84 (m, 6H), 3.74 - 3.66 (m, 2H), 1.97 (s, 3H), 1.33 (d, *J* = 7.2 Hz, 3H); ¹³C NMR (101 MHz, 20% D₂O, pH = 5) δ 176.4, 176.2, 174.5, 172.3, 172.1, 171.2, 50.1, 43.3, 42.7, 42.7, 42.6, 21.8, 16.5.



δ/ppm

Figure S1. Stacked ¹H-NMR spectra of an aqueous reaction (0.5 mL, H₂O/D₂O, 4:1) of Ac-Ala **2** (100 mM), glycine **3a** (50 mM), methyl isonitrile **1** (~ 130 mM) at pH 5, 23 °C. A) Reaction mixture at t = 0; B) Reaction mixture at t = 15 days; C) Synthetic standard of Ac-Ala-Gly **4a**; D) Synthetic standard of Ac-Ala-Gly-Gly **4b**. The yield of incorporation of glycine **3a** to peptide products **4a** and **4c** was 62 %.



4.5 4.4 4.3 4.2 4.1 4.0 3.9 3.8 3.7 3.6 3.5 3.4 3.3 3.2 3.1 3.0 2.9 2.8 2.7 2.6 2.5 2.4 2.3 2.2 2.1 2.0 1.9 1.8 1.7 1.6 1.5 1.4 1.3 1.2 1.1 1.0 δ/ ppm

Figure S2. Stacked ¹H-NMR spectra of an aqueous reaction (0.5 mL, H₂O/D₂O, 4:1) of Ac-Ala **2** (100 mM), β -alanine **3b** (50 mM), methyl isonitrile **1** (~ 130 mM) at pH 5, 23 °C. A) Reaction mixture at t = 0; B) Reaction mixture at t = 15 days; C) Synthetic standard of Ac-Ala- β -Ala **4b**. The yield of incorporation of β -alanine **3b** to amide product **4b** was 81 %.



4.5 4.4 4.3 4.2 4.1 4.0 3.9 3.8 3.7 3.6 3.5 3.4 3.3 3.2 3.1 3.0 2.9 2.8 2.7 2.6 2.5 2.4 2.3 2.2 2.1 2.0 1.9 1.8 1.7 1.6 1.5 1.4 1.3 1.2 1.1 1.0 δ/ ppm

Figure S3. Stacked ¹H-NMR spectra of an aqueous reaction (0.5 mL, H₂O/D₂O, 4:1) of Ac-Ala **2** (100 mM), glycylglycine **3c** (50 mM), methyl isonitrile **1** (~ 130 mM) at pH 5, 23 °C. A) Reaction mixture at t = 0; B) Reaction mixture at t = 15 days; C) Synthetic standard of Ac-Ala-Gly-Gly **4c**; D) Synthetic standard of Ac-Ala-Gly-Gly-Gly-Gly-Gly **4i**. The yield of incorporation of glycylglycine **3** to peptide products **4c** and **4i** was 72 %.



Figure S4. Stacked ¹H-NMR spectra of an aqueous reaction (0.5 mL, H₂O/D₂O, 4:1) of Ac-Ala **2** (100 mM), glycinamide **3d** (50 mM), methyl isonitrile **1** (~ 130 mM) at pH 5, 23 °C. A) Reaction mixture at t = 0; B) Reaction mixture at t = 15 days; C) Synthetic standard of Ac-Ala-Gly-NH₂ **4d**. The yield of incorporation of glycinamide **3d** to amide product **4d** was 69 %.



4.5 4.4 4.3 4.2 4.1 4.0 3.9 3.8 3.7 3.6 3.5 3.4 3.3 3.2 3.1 3.0 2.9 2.8 2.7 2.6 2.5 2.4 2.3 2.2 2.1 2.0 1.9 1.8 1.7 1.6 1.5 1.4 1.3 1.2 1.1 1.0 δ/ppm

Figure S5. Stacked ¹H-NMR spectra of an aqueous reaction (0.5 mL, H₂O/D₂O, 4:1) of Ac-Ala **2** (100 mM), glycine nitrile **3e** (50 mM), methyl isonitrile **1** (~ 130 mM) at pH 5, 23 °C. A) Reaction mixture at t = 0; B) Reaction mixture at t = 15 days; C) Synthetic standard of Ac-Ala-Gly-CN **4e**. The yield of incorporation of glycine nitrile **3e** to amide product **4e** was 86 %.



4.5 4.4 4.3 4.2 4.1 4.0 3.9 3.8 3.7 3.6 3.5 3.4 3.3 3.2 3.1 3.0 2.9 2.8 2.7 2.6 2.5 2.4 2.3 2.2 2.1 2.0 1.9 1.8 1.7 1.6 1.5 1.4 1.3 1.2 1.1 δ/ppm

Figure S6. Stacked ¹H-NMR spectra of an aqueous reaction (0.5 mL, H₂O/D₂O, 4:1) of Ac-Ala **2** (100 mM), methylammonium chloride **3f** (50 mM), methyl isonitrile **1** (\sim 130 mM) at pH 5, 23 °C. A) Reaction mixture at t = 0; B) Reaction mixture at t = 15 days; C) Synthetic standard of Ac-Ala-NHCH₃ **4f**. The yield of incorporation of methylamine **3f** to amide product **4f** was 47 %.



 δ / ppm

Figure S7. Stacked ¹H-NMR spectra of an aqueous reaction (0.5 mL, H₂O/D₂O, 4:1) of Ac-Ala **2** (100 mM), ammonium chloride **3g** (50 mM), methyl isonitrile **1** (~ 130 mM) at pH 5, 23 °C. A) Reaction mixture at t = 0; B) Reaction mixture at t = 15 days; C) Synthetic standard of Ac-Ala-NH₂ **4g** (with minor impurity of Ac-Ala **2**). The yield of incorporation of ammonia **3g** to amide product **4g** was 17 %.



Figure S8. Stacked ¹H-NMR spectra of an aqueous reaction (0.5 mL, H₂O/D₂O, 4:1) of Ac-Ala **2** (100 mM), glycine **3a** (50 mM), methyl isonitrile **1** (~ 130 mM) at pH 4, 23 °C. A) Reaction mixture at t = 0; B) Reaction mixture at t = 2 days; C) Synthetic standard of Ac-Ala-Gly **4a**. The yield of incorporation of glycine **3a** to peptide products **4a** was 58 %.



Figure S9. Stacked ¹H-NMR spectra of an aqueous reaction (0.5 mL, H₂O/D₂O, 4:1) of Ac-Ala **2** (100 mM), β -alanine **3b** (50 mM), methyl isonitrile **1** (~ 130 mM) at pH 4, 23 °C. A) Reaction mixture at t = 0; B) Reaction mixture at t = 2 days. The yield of incorporation of β -alanine **3b** to amide product **4b** was 47 %.



δ / ppm

Figure S10. Stacked ¹H-NMR spectra of an aqueous reaction (0.5 mL, H₂O/D₂O, 4:1) of Ac-Ala **2** (100 mM), glycylglycine **3c** (50 mM), methyl isonitrile **1** (~ 130 mM) at pH 4, 23 °C. A) Reaction mixture at t = 0; B) Reaction mixture at t = 2 days; C) Synthetic standard of Ac-Ala-Gly-Gly **4c**. The yield of incorporation of glycylglycine **3c** to peptide products **4c** was 19 %.



4.4 4.3 4.2 4.1 4.0 3.9 3.8 3.7 3.6 3.5 3.4 3.3 3.2 3.1 3.0 2.9 2.8 2.7 2.6 2.5 2.4 2.3 2.2 2.1 2.0 1.9 1.8 1.7 1.6 1.5 1.4 1.3 1.2 1.1 1.0 δ/ ppm

Figure S11. Stacked ¹H-NMR spectra of an aqueous reaction (0.5 mL, H₂O/D₂O, 4:1) of Ac-Ala **2** (100 mM), glycinamide **3d** (50 mM), methyl isonitrile **1** (~ 130 mM) at pH 4, 23 °C. A) Reaction mixture at t = 0; B) Reaction mixture at t = 2 days; C) Synthetic standard of Ac-Ala-Gly-NH₂ **4d**. The yield of incorporation of glycinamide **3d** to amide product **4d** was 20 %.



4.6 4.5 4.4 4.3 4.2 4.1 4.0 3.9 3.8 3.7 3.6 3.5 3.4 3.3 3.2 3.1 3.0 2.9 2.8 2.7 2.6 2.5 2.4 2.3 2.2 2.1 2.0 1.9 1.8 1.7 1.6 1.5 1.4 1.3 1.2 1.1 δ/ppm

Figure S12. Stacked ¹H-NMR spectra of an aqueous reaction (0.5 mL, H₂O/D₂O, 4:1) of Ac-Ala **2** (100 mM), methylammonium chloride **3f** (50 mM), methyl isonitrile **1** (\sim 130 mM) at pH 4, 23 °C. A) Reaction mixture at t = 0; B) Reaction mixture at t = 2 days. No amide product was detected. C) Synthetic standard of Ac-Ala-NHCH₃ **4f**.



4.5 4.4 4.3 4.2 4.1 4.0 3.9 3.8 3.7 3.6 3.5 3.4 3.3 3.2 3.1 3.0 2.9 2.8 2.7 2.6 2.5 2.4 2.3 2.2 2.1 2.0 1.9 1.8 1.7 1.6 1.5 1.4 1.3 1.2 1.1 δ/ ppm

Figure S13. Stacked ¹H-NMR spectra of an aqueous reaction (0.5 mL, H₂O/D₂O, 4:1) of Ac-Ala **2** (100 mM), ammonium chloride **3g** (50 mM), methyl isonitrile **1** (~ 130 mM) at pH 4, 23 °C. A) Reaction mixture at t = 0; B) Reaction mixture at t = 2 days. No amide product was detected. C) Synthetic standard of Ac-Ala-NH₂ **4g** (with minor impurity of Ac-Ala **2**).



δ/ppm

Figure S14. Stacked ¹H-NMR spectra of an aqueous reaction (0.5 mL, H₂O/D₂O, 4:1) of Ac-Ala **2** (100 mM), glycine nitrile **3e** (50 mM), methyl isonitrile **1** (~ 130 mM) at pH 4, 23 °C. A) Reaction mixture at t = 0; B) Reaction mixture at t = 2 days; C) Synthetic standard of Ac-Ala-Gly-CN **4e**. The yield of incorporation of glycine nitrile **3e** to amide product **4e** was 100 %.



4.6 4.5 4.4 4.3 4.2 4.1 4.0 3.9 3.8 3.7 3.6 3.5 3.4 3.3 3.2 3.1 3.0 2.9 2.8 2.7 2.6 2.5 2.4 2.3 2.2 2.1 2.0 1.9 1.8 1.7 1.6 1.5 1.4 1.3 1.2 δ/ppm

Figure S15. Stacked ¹H-NMR spectra of an aqueous reaction (0.5 mL, H₂O/D₂O, 4:1) of Ac-Ala 2 (100 mM), glycine **3a** (50 mM), methyl isonitrile **1** (~ 130 mM) at pH 3, 23 °C. A) Reaction mixture at t = 0; B) Reaction mixture at t = 6 hours; C) Synthetic standard of Ac-Ala-Gly **4a**. The yield of incorporation of glycine **3a** to peptide product **4a** was 46 %.



δ / ppm

Figure S16. Stacked ¹H-NMR spectra of an aqueous reaction (0.5 mL, H₂O/D₂O, 4:1) of Ac-Ala **2** (100 mM), β -alanine **3b** (50 mM), methyl isonitrile **1** (~ 130 mM) at pH 3, 23 °C. A) Reaction mixture at t = 0; B) Reaction mixture at t = 6 hours. C) Synthetic standard of Ac-Ala-Gly **4b**. The yield of incorporation of β -alanine **3b** to peptide product **4b** was 5 %.



4.5 4.4 4.3 4.2 4.1 4.0 3.9 3.8 3.7 3.6 3.5 3.4 3.3 3.2 3.1 3.0 2.9 2.8 2.7 2.6 2.5 2.4 2.3 2.2 2.1 2.0 1.9 1.8 1.7 1.6 1.5 1.4 1.3 1.2 δ/ ppm

Figure S17. Stacked ¹H-NMR spectra of an aqueous reaction (0.5 mL, H₂O/D₂O, 4:1) of Ac-Ala **2** (100 mM), glycine nitrile **3e** (50 mM), methyl isonitrile **1** (~ 130 mM) at pH 3, 23 °C. A) Reaction mixture at t = 0; B) Reaction mixture at t = 6 hours; C) Synthetic standard of Ac-Ala-Gly-CN **4e**. The yield of incorporation of glycine nitrile **3e** to amide product **4e** was 30 %.



4.5 4.4 4.3 4.2 4.1 4.0 3.9 3.8 3.7 3.6 3.5 3.4 3.3 3.2 3.1 3.0 2.9 2.8 2.7 2.6 2.5 2.4 2.3 2.2 2.1 2.0 1.9 1.8 1.7 1.6 1.5 1.4 1.3 1.2 1.1 δ/ppm

Figure S18. Stacked ¹H-NMR spectra of an aqueous reaction (0.5 mL, H₂O/D₂O, 4:1) of Ac-Ala **2** (100 mM), arginine (50 mM), methyl isonitrile **1** (~ 130 mM) at pH 4, 23 °C. A) Reaction mixture at t = 0; B) Reaction mixture at t = 2 days. Products were tentatively assigned, and the yield of incorporation of arginine to peptide products was 64 %.



4.6 4.5 4.4 4.3 4.2 4.1 4.0 3.9 3.8 3.7 3.6 3.5 3.4 3.3 3.2 3.1 3.0 2.9 2.8 2.7 2.6 2.5 2.4 2.3 2.2 2.1 2.0 1.9 1.8 1.7 1.6 1.5 1.4 1.3 1.2 1.1 1.0 δ/ppm

Figure S19. Stacked ¹H-NMR spectra of an aqueous reaction (0.5 mL, H₂O/D₂O, 4:1) of Ac-Ala **2** (100 mM), serine (50 mM), methyl isonitrile **1** (~ 130 mM) at pH 4, 23 °C. A) Reaction mixture at t = 0; B) Reaction mixture at t = 2 days. Products were tentatively assigned, and the yield of incorporation of serine to peptide products was 44 %.



Figure S20. Stacked ¹H-NMR spectra of an aqueous reaction (0.5 mL, H₂O/D₂O, 4:1) of Ac-Ala **2** (100 mM), valine (50 mM), methyl isonitrile **1** (~ 130 mM) at pH 4, 23 °C. A) Reaction mixture at t = 0; B) Reaction mixture at t = 2 days. Products were tentatively assigned, and the yield of incorporation of valine to peptide products was 66 %.



δ / ppm

Figure S21. Stacked ¹H-NMR spectra of an aqueous reaction (0.5 mL, H_2O/D_2O , 4:1) of Ac-Ala 2 (100 mM), proline (50 mM), methyl isonitrile 1 (~ 130 mM) at pH 4, 23 °C. A) Reaction mixture at t = 0; B) Reaction mixture at t = 2 days. Products were tentatively assigned, and the yield of incorporation of proline to peptide products was 52 %.



δ/ppm

Figure S22. Stacked ¹H-NMR spectra of an aqueous reaction (0.5 mL, H₂O/D₂O, 4:1) of Ac-Ala **2** (100 mM), glycine **3a** (25 mM), β-alanine **2b** (25 mM), glycylglycine **3c** (25 mM), glycinamide **3d** (25 mM), glycinenitrile **3e** (25 mM), methylamine **3f** (25 mM), ammonia **3g** (25 mM) and methyl isonitrile **1** (~ 130 mM) at pH 3, 23 °C. A) Reaction mixture at t = 0; B) Reaction mixture at t = 6 hours; C) Synthetic standard of Ac-Ala-Gly **4a**; D) Synthetic standard of Ac-Ala-Gly-CN **4b**. E) Synthetic standard of Ac-Ala-Gly-CN **4e**. The yield of incorporation of glycine **3a** to **4a** and glycinenitrile **3e** to **4e** were 55 % and 42 % respectively, **4b** was not observed.



4.4 4.3 4.2 4.1 4.0 3.9 3.8 3.7 3.6 3.5 3.4 3.3 3.2 3.1 3.0 2.9 2.8 2.7 2.6 2.5 2.4 2.3 2.2 2.1 2.0 1.9 1.8 1.7 1.6 1.5 1.4 1.3 1.2 1.1 1.0 0.9 0.8 0.7 δ / ppm

Figure S23. Stacked ¹H-NMR spectra of an aqueous reaction (0.5 mL, H₂O/D₂O, 4:1) of Ac-Ala 2 (20 mM), glycine **3a** (100 mM), methyl isonitrile **1** (\sim 100 mM) at pH 3, 23 °C. A) Reaction mixture at t = 6 hours, after adjusting the pH of the solution to 5; B) Synthetic standard of Ac-Ala-Gly **4a**; C) Synthetic standard of Ac-Ala-Gly-Gly **4c**.



Figure S24. Stacked ¹H-NMR spectra of an aqueous reaction (0.5 mL, H₂O/D₂O, 4:1) of Ac-Ala **2** (20 mM), glycine **3a** (100 mM), methyl isonitrile **1** (~ 100 mM) at pH 4, 23 °C. A) Reaction mixture at t = 24 hours, after adjusting the pH of the solution to 5; B) Synthetic standard of Ac-Ala-Gly **4a**; C) Synthetic standard of Ac-Ala-Gly-Gly **4c**.



4.5 4.4 4.3 4.2 4.1 4.0 3.9 3.8 3.7 3.6 3.5 3.4 3.3 3.2 3.1 3.0 2.9 2.8 2.7 2.6 2.5 2.4 2.3 2.2 2.1 2.0 1.9 1.8 1.7 1.6 1.5 1.4 1.3 1.2 δ/ ppm

Figure S25. Stacked ¹H-NMR spectra of an aqueous reaction (0.5 mL, H₂O/D₂O, 4:1) of Ac-Ala **2** (20 mM), glycine **3a** (100 mM), methyl isonitrile **1** (~ 100 mM) at pH 5, 23 °C. A) Reaction mixture at t = 0; B) Reaction mixture at t = 15 days; C) Synthetic standard of Ac-Ala-Gly **4a**; D) Synthetic standard of Ac-Ala-Gly-Gly **4c**; E) Synthetic standard of Ac-Ala-Gly-Gly **4h**.



Figure S26. Stacked ¹H-NMR spectra of an aqueous reaction (0.5 mL, H₂O/D₂O, 4:1) of Ac-Ala **2** (100 mM), glycolic acid **8** (50 mM), methyl isonitrile **1** (~ 130 mM) at pH 4, 23 °C. A) Reaction mixture at t = 0; B) Reaction mixture at t = 2 days. The yield of incorporation of glycolic acid **8** to ester product **9** was 88 %.



Figure S27. Stacked ¹H-NMR spectra of an aqueous reaction (0.5 mL, H₂O/D₂O, 4:1) of Ac-Ala **2** (100 mM), glycolic acid **8** (50 mM), methyl isonitrile **1** (\sim 130 mM) at pH 3, 23 °C. A) Reaction mixture at t = 0; B) Reaction mixture at t = 6 hours. The yield of incorporation of glycolic acid **8** to ester product **9** was 66 %.



.6 4.5 4.4 4.3 4.2 4.1 4.0 3.9 3.8 3.7 3.6 3.5 3.4 3.3 3.2 3.1 3.0 2.9 2.8 2.7 2.6 2.5 2.4 2.3 2.2 2.1 2.0 1.9 1.8 1.7 1.6 1.5 1.4 1.3 1.2 δ/ppm

Figure S28. Stacked ¹H-NMR spectra of an aqueous reaction (0.5 mL, H₂O/D₂O, 4:1) of Ac-Ala **2** (100 mM), methyl glycolate **11** (50 mM), methyl isonitrile **1** (~ 130 mM) at pH 4, 23 °C. A) Reaction mixture at t = 0; B) Reaction mixture at t = 2 days. No ester product **12** was detected. n. d. : not detected.



Figure S29. ¹H-NMR spectrum of an aqueous reaction (0.5 mL, H_2O/D_2O , 4:1) of glycine **3a** (100 mM), methyl isonitrile **1** (~ 130 mM) at pH 4, 23 °C after 2 days.



Figure S30. Stacked ¹H-NMR spectra of an aqueous reaction (0.5 mL, H₂O/D₂O, 4:1) of glycine **3a** (100 mM), methyl glycolate **11** (50 mM), methyl isonitrile **1** (\sim 130 mM) at pH 4, 23 °C. A) Reaction mixture at t = 2 days; B) Standard of *N*-methyl formamide **15**.



Figure S31. Stacked ¹³C-NMR spectra of an aqueous reaction (0.5 mL, H₂O/D₂O, 4:1) of glycine **3a** (100 mM), methyl isonitrile **1** (~ 130 mM) at pH 4, 23 °C after 2 days. A) Reaction mixture at t = 2 days; B) Standard of *N*-methyl formamide **15**.



Figure S32. HSQC spectrum of an aqueous reaction (0.5 mL, H_2O/D_2O , 4:1) of glycine **3a** (100 mM), methyl isonitrile **1** (~ 130 mM) at pH 4, 23 °C after 2 days.



Figure S33. HMBC spectrum of an aqueous reaction (0.5 mL, H_2O/D_2O , 4:1) of glycine 3a (100 mM), methyl isonitrile 1 (~ 130 mM) at pH 4, 23 °C after 2 days.

Summary of the assignment of the side product **13** based on spectra in Figure S29-S33:

¹H NMR (400 MHz, 20 % D₂O, pH = 4) δ 7.96 (s, 1H, 15), 7.73 (dt, *J* = 13.8, 7.4 Hz, 1H, 13), 3.97 - 3.92 (m, 2H, 13-*trans*), 3.83 - 3.79 (m, 2H, 13-*cis*), 3.50 (s, 2H, 3a), 3.05 (d, *J* = 4.2 Hz, 3H, 13-*cis*), 2.91 (d, *J* = 4.9 Hz, 3H, 13-*trans*), 2.83 (d, *J* = 4.5 Hz, 15-*cis*), 2.70 (d, *J* = 4.8 Hz, 15-*trans*); ¹³C NMR (101 MHz, 20 % D₂O, pH = 4) δ 175.0 (13-*trans*), 172.9 (13-*cis*), 172.39 (3a), 168.1 (15-*cis*), 165.0 (15-*trans*), 156.5 (13-*trans*), 155.2 (13-*cis*), 49.1 (13-*trans*), 44.9 (13-*cis*), 41.6 (3a), 33.2 (13-*cis*), 28.0 (13-*trans*), 27.9 (15-*cis*), 24.6 (15-*trans*).



Figure S34. Stacked ¹H-NMR spectra of an aqueous reaction (0.5 mL, H₂O/D₂O, 4:1) of *N*-formyl-Ala (100 mM), glycine **3a** (50 mM), methyl isonitrile **1** (\sim 130 mM) at pH 5, 23 °C. A) Reaction mixture at t = 0; B) Reaction mixture at t = 15 days.



Figure S35. ¹H-NMR and ¹³C-NMR spectra of 4b in 20 % D₂O/H₂O.



Figure S36. ¹H-NMR and ¹³C-NMR spectra of 4c in 20 % D₂O/H₂O.



Figure S37. ¹H-NMR and ¹³C-NMR spectra of 4d in 20 % D₂O/H₂O.



Figure S38. ¹H-NMR and ¹³C-NMR spectra of 4e in 20 % D₂O/H₂O.



Figure S39. ¹H-NMR and ¹³C-NMR spectra of 4f in 20 % D₂O/H₂O.



Figure S40. ¹H-NMR and ¹³C-NMR spectra of 4g in 20 % D₂O/H₂O.



Figure S41. ¹H-NMR and ¹³C-NMR spectra of 4h in 20 % D₂O/H₂O.



Figure S42. ¹H-NMR and ¹³C-NMR spectra of 4i in 20 % D_2O/H_2O .



Figure S43. ¹H-NMR and ¹³C-NMR spectra of Ac-Ala-Gly-Gly-OMe in 20 % D_2O/H_2O .

References

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