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. $^1$H-, and $^{13}$C-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 H$_2$O/D$_2$O mixtures were analysed using HOD suppression to collect $^1$H-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, quartriplet, multiplet, and broad signals, respectively. The yields of conversion were determined by relative integration of the signals in the $^1$H-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 H$_2$SO$_4$ solution.

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

An aqueous solution (0.5 mL, H$_2$O/D$_2$O, 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 µL, concentration in theory would be 200 mM, ~ 130 mM in fact, due to its volatility, based on $^1$H-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 $^1$H-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 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 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 µL, ~ 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 $^1$H-NMR to have better resolution of peaks in $^1$H-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% D$_2$O) and checked by $^1$H-NMR spectroscopy. After all the Ac-Ala was converted, the pH of reaction mixture was adjusted to 5. Then the mixture was passed though 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). $^1$H NMR (400 MHz, 20 % D$_2$O, pH = 1) $\delta$ = 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); $^{13}$C NMR (101 MHz, 20 % D$_2$O, pH = 1) $\delta$ = 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% D$_2$O) and checked by $^1$H-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 % D$_2$O), and the solution was adjusted to pH 12. The mixed was monitored by $^1$H-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 % D$_2$O), the
solution was adjusted to pH 5 and monitored by NMR spectroscopy at 23 °C. \(^1\)H NMR (400 MHz, 20 % D\(_2\)O, pH = 5) \(\delta\) 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); \(^{13}\)C NMR (101 MHz, 20% D\(_2\)O, pH = 5) \(\delta\) 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 % D\(_2\)O), and the solution was adjusted to pH 12. The mixed was monitored by \(^1\)H-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 % D\(_2\)O), the solution was adjusted to pH 5 and monitored by NMR spectroscopy at 23 °C. \(^1\)H-NMR (400 Hz, 20 % D\(_2\)O, pH = 5) \(\delta\) = 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); \(^{13}\)C-NMR (101 Hz, 20 % D\(_2\)O, pH = 5) \(\delta\) = 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-NH\(_2\) 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 µL of the reaction mixture was mixed with 450 µL of water (with 20% D\(_2\)O) and checked by \(^1\)H-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 % D\(_2\)O/H\(_2\)O and the solution was monitored by NMR spectroscopy. \(^1\)H NMR (400 MHz, 20 % D\(_2\)O) \(\delta\) 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); \(^{13}\)C NMR (101 MHz, 20 % D\(_2\)O) \(\delta\) 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 through 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 µL of the reaction mixture was mixed with 450 µL 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 through 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 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 through 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 % D$_2$O/H$_2$O and the solution was monitored by NMR spectroscopy. $^1$H NMR (400 MHz, 20 % D$_2$O) $\delta$ 4.19 (qd, $J = 7.2, 6.0$ Hz, 1H), 1.96 (s, 3H), 1.32 (d, $J = 7.3$ Hz, 3H); $^{13}$C NMR (101 MHz, 20 % D$_2$O) $\delta$ 178.3, 174.2, 49.7, 21.8, 16.8.

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

Ac-Ala-Gly$^1$ (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 $\mu$L of the reaction mixture was mixed with 450 $\mu$L of water (with 20% D$_2$O) and checked by $^1$H-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 through 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-Gly-OMe) was dissolved in 0.5 mL water (with 20 % D$_2$O), and the solution was adjusted to pH 12. The mixed was monitored by $^1$H-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 % D$_2$O), the solution was adjusted to pH 5 and monitored by NMR spectroscopy at 23 °C. $^1$H NMR (400 MHz, 20 % D$_2$O, pH = 5) $\delta$ 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); $^{13}$C (101 MHz, 20 % D$_2$O, pH = 5) $\delta$ 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 $\mu$L of the reaction mixture was mixed with 450 $\mu$L of water (with 20% D$_2$O) and checked by $^1$H-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% D$_2$O) and checked by $^1$H-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 % D$_2$O), and the solution was adjusted to pH 12. The mixed was monitored by $^1$H-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 % D$_2$O), the solution was adjusted to pH 5 and monitored by NMR spectroscopy at 23 °C. $^1$H NMR (400 MHz, 20 % D$_2$O, 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); $^{13}$C NMR (101 MHz, 20% D$_2$O, 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.
Figure S1. Stacked $^1$H-NMR spectra of an aqueous reaction (0.5 mL, H$_2$O/D$_2$O, 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 %.
Figure S2. Stacked $^1$H-NMR spectra of an aqueous reaction (0.5 mL, H$_2$O/D$_2$O, 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%.
Figure S3. Stacked $^1$H-NMR spectra of an aqueous reaction (0.5 mL, H$_2$O/D$_2$O, 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 to peptide products 4c and 4i was 72%.
Figure S4. Stacked $^1$H-NMR spectra of an aqueous reaction (0.5 mL, H$_2$O/D$_2$O, 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-NH$_2$ 4d. The yield of incorporation of glycinamide 3d to amide product 4d was 69%.
Figure S5. Stacked $^1$H-NMR spectra of an aqueous reaction (0.5 mL, H$_2$O/D$_2$O, 4:1) of Ac-Ala 2 (100 mM), glycine nitrile 3e (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-Gly-CN 4e. The yield of incorporation of glycine nitrile 3e to amide product 4e was 86 %.
Figure S6. Stacked $^1$H-NMR spectra of an aqueous reaction (0.5 mL, H$_2$O/D$_2$O, 4:1) of Ac-Ala 2 (100 mM), methylammonium chloride 3f (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-NHCH$_3$ 4f. The yield of incorporation of methylamine 3f to amide product 4f was 47 %.
Figure S7. Stacked $^1$H-NMR spectra of an aqueous reaction (0.5 mL, H$_2$O/D$_2$O, 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-NH$_2$ 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 %. 

$$\text{Ac-Ala, 2} \quad 100 \text{ mM}$$

$$\text{Amine, 3a} \quad 50 \text{ mM}$$

$$\text{Me=N} \quad 1. \sim 130 \text{ mM}$$

$$\text{pH 4, H}_2\text{O}$$

$$\text{2 days, 23 °C}$$

$$\text{4a, ~29 mM}$$

$$\text{13, ~6 mM}$$
**Figure S9.** Stacked $^1$H-NMR spectra of an aqueous reaction (0.5 mL, H$_2$O/D$_2$O, 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%.
Figure S10. Stacked $^1$H-NMR spectra of an aqueous reaction (0.5 mL, H$_2$O/D$_2$O, 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%.
Figure S11. Stacked $^1$H-NMR spectra of an aqueous reaction (0.5 mL, H$_2$O/D$_2$O, 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-NH$_2$ 4d. The yield of incorporation of glycinamide 3d to amide product 4d was 20 %.
Figure S12. Stacked $^1$H-NMR spectra of an aqueous reaction (0.5 mL, H$_2$O/D$_2$O, 4:1) of Ac-Ala 2 (100 mM), methylammonium chloride 3f (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-NHCH$_3$ 4f. No amide product was detected.
Figure S13. Stacked $^{1}$H-NMR spectra of an aqueous reaction (0.5 mL, H$_2$O/D$_2$O, 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; C) Synthetic standard of Ac-Ala-NH$_2$ 4g (with minor impurity of Ac-Ala 2). No amide product was detected.
**Figure S14.** Stacked $^1$H-NMR spectra of an aqueous reaction (0.5 mL, H$_2$O/D$_2$O, 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 %.
Figure S15. Stacked $^1$H-NMR spectra of an aqueous reaction (0.5 mL, H$_2$O/D$_2$O, 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 %.
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%.
Figure S17. Stacked $^1$H-NMR spectra of an aqueous reaction (0.5 mL, H$_2$O/D$_2$O, 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%.
Figure S18. Stacked $^1$H-NMR spectra of an aqueous reaction (0.5 mL, H$_2$O/D$_2$O, 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 %.
Figure S19. Stacked $^1$H-NMR spectra of an aqueous reaction (0.5 mL, H$_2$O/D$_2$O, 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 $^1$H-NMR spectra of an aqueous reaction (0.5 mL, H$_2$O/D$_2$O, 4:1) of Ac-Ala 2 (100 mM), valine (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. Products were tentatively assigned, and the yield of incorporation of valine to peptide products was 66%.
Figure S21. Stacked $^1$H-NMR spectra of an aqueous reaction (0.5 mL, H$_2$O/D$_2$O, 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 %.
Figure S22. Stacked $^1$H-NMR spectra of an aqueous reaction (0.5 mL, H$_2$O/D$_2$O, 4:1) of Ac-Ala 2 (100 mM), glycine 3a (25 mM), β-alanine 2b (25 mM), glycylglycine 3c (25 mM), glycaminide 3d (25 mM), glycinenitrile 3e (25 mM), methyamine 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.
Figure S23. Stacked $^1$H-NMR spectra of an aqueous reaction (0.5 mL, H$_2$O/D$_2$O, 4:1) of Ac-Ala 2 (20 mM), glycine 3a (100 mM), methyl isonitrile 1 (~ 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 $^1$H-NMR spectra of an aqueous reaction (0.5 mL, H$_2$O/D$_2$O, 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.
Figure S25. Stacked $^1$H-NMR spectra of an aqueous reaction (0.5 mL, H$_2$O/D$_2$O, 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-Gly 4h.
**Figure S26.** Stacked $^1$H-NMR spectra of an aqueous reaction (0.5 mL, H$_2$O/D$_2$O, 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 $^1$H-NMR spectra of an aqueous reaction (0.5 mL, H$_2$O/D$_2$O, 4:1) of Ac-Ala 2 (100 mM), glycolic acid 8 (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. The yield of incorporation of glycolic acid 8 to ester product 9 was 66 %.
Figure S28. Stacked $^1$H-NMR spectra of an aqueous reaction (0.5 mL, H$_2$O/D$_2$O, 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. $^1$H-NMR spectrum of an aqueous reaction (0.5 mL, H$_2$O/D$_2$O, 4:1) of glycine 3a (100 mM), methyl isonitrile 1 (~ 130 mM) at pH 4, 23 °C after 2 days.
Figure S30. Stacked $^1$H-NMR spectra of an aqueous reaction (0.5 mL, $\text{H}_2\text{O}/\text{D}_2\text{O}, 4:1$) of glycine 3a (100 mM), methyl glycolate 11 (50 mM), methyl isonitrile 1 (~ 130 mM) at pH 4, 23 °C. A) Reaction mixture at $t = 2$ days; B) Standard of $N$-methyl formamide 15.
Figure S31. Stacked $^{13}$C-NMR spectra of an aqueous reaction (0.5 mL, H$_2$O/D$_2$O, 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$_2$O/D$_2$O, 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$_2$O/D$_2$O, 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:

$^1$H NMR (400 MHz, 20 % D$_2$O, 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); $^{13}$C NMR (101 MHz, 20 % D$_2$O, 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 $^1$H-NMR spectra of an aqueous reaction (0.5 mL, H$_2$O/D$_2$O, 4:1) of N-formyl-Ala (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.
Figure S35. $^1$H-NMR and $^{13}$C-NMR spectra of 4b in 20 % D$_2$O/H$_2$O.
Figure S36. $^1$H-NMR and $^{13}$C-NMR spectra of 4c in 20 % D$_2$O/H$_2$O.
**Figure S37.** $^1$H-NMR and $^{13}$C-NMR spectra of 4d in 20 % $\text{D}_2\text{O}/\text{H}_2\text{O}$. 
Figure S38. $^1$H-NMR and $^{13}$C-NMR spectra of 4e in 20% D$_2$O/H$_2$O.
Figure S39. $^1$H-NMR and $^{13}$C-NMR spectra of 4f in 20 % D$_2$O/H$_2$O.
Figure S40. $^1$H-NMR and $^{13}$C-NMR spectra of 4g in 20 % D$_2$O/H$_2$O.
Figure S41. $^1$H-NMR and $^{13}$C-NMR spectra of 4h in 20 % D$_2$O/H$_2$O.
Figure S42. $^1$H-NMR and $^{13}$C-NMR spectra of 4i in 20 % D$_2$O/H$_2$O.
Figure S43. $^1$H-NMR and $^{13}$C-NMR spectra of Ac-Ala-Gly-Gly-OMe in 20 % D$_2$O/H$_2$O.
References

(1) Z., Liu, L.-F. Wu, J. Xu, C. Bonfio, D. A. Russell and J. D. Sutherland, Harnessing chemical energy for the activation and joining of prebiotic building blocks. *Nat. Chem.* 2020, **12**, 1023-1028.