

# **A Reassessment of Prebiotically Relevant Chemical Agents for the Activation of $\alpha$ -Amino Acids and Peptides**

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**SUPPORTING INFORMATION**

## Synthesis of starting materials and reagents

### **H-Tyr(Me)-L-Ala-OH: 1-LL**

A mixture of Boc-L-Tyr(Me)-OH (1 g, 3.38 mmol) and HOBt (0.56 g, 3.71 mmol) was stirred in CH<sub>2</sub>Cl<sub>2</sub> (5 mL) for 5 min vigorously. Then the solution was cooled to 0°C and dicyclohexylcarbodiimide (DCC, 0.73 g, 3.54 mmol) was added. The mixture was stirred at 0°C for 30 min. The solid dicyclohexylurea was filtered off, washed with CH<sub>2</sub>Cl<sub>2</sub> and the filtrate was collected. The solution was concentrated under vacuum. Then the Boc-L-Tyr(Me)-OBt residue was subsequently reacted in DMF (3 mL) with L-Alanine tert-butyl ester hydrochloride salt (0.675 g, 3.7 mmol) in the presence of DIEA (1.17 mL, 6.76 mmol). The mixture was stirred overnight at *r.t.* Then water (20 mL) was added and the mixture was extracted with ethyl acetate (2 × 50 mL). Then the organic layer was washed with concentrated Na<sub>2</sub>CO<sub>3</sub>, 1 M KHSO<sub>4</sub>, brine, and dried over Na<sub>2</sub>SO<sub>4</sub> and finally concentrated under reduced pressure. The Boc and tert-butyl protecting groups were removed from the oily residue (1.006 g) by treatment with TFA/H<sub>2</sub>O (90:10) (10 mL) for 30 min. The solution was concentrated under vacuum. The oily residue was treated with 15 mL cold ethyl ether then it was kept at 4°C overnight. The white precipitate was filtered and washed with ethyl ether and then dried (600 mg); <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ<sub>H</sub> / ppm: 8.83 (t, *J* = 12.0 Hz, 1 H), 7.22 (d, *J* = 8.7 Hz, 2 H), 6.94 – 6.82 (m, 2 H), 4.36 – 4.21 (m, 1 H), 3.97 (dd, *J* = 7.9, 5.2 Hz, 1 H), 3.74 (s, 3 H), 3.08 (dd, *J* = 14.2, 5.1 Hz, 1 H), 2.87 (dd, *J* = 14.3, 8.0 Hz, 1 H), 1.32 (t, *J* = 8.3 Hz, 3 H); <sup>13</sup>C NMR (101 MHz, DMSO-*d*<sub>6</sub>) δ<sub>C</sub> / ppm: 174.19 (s), 168.31 (s), 158.84 (s), 131.14 (s), 126.87 – 126.67 (m), 114.44 (s), 55.52 (s), 53.79 (s), 48.17 (s), 36.53 (s), 17.70 (s); HPLC (Method A): retention time 9.6 min; HRMS (ESI positive mode): calcd for C<sub>13</sub>H<sub>19</sub>N<sub>2</sub>O<sub>4</sub><sup>+</sup> [M+H<sup>+</sup>] 267.1345; found 267.1350.

### **H-Tyr(Me)-D-Ala-OH: 1-LD**

A mixture of Boc-L-Tyr(Me)-OH (0.25 g, 0.84 mmol) and HOBt (0.14 g, 0.93 mmol) was stirred in CH<sub>2</sub>Cl<sub>2</sub> (5 mL) for 5 min vigorously. After 5 min the solution was cooled down to 0°C. DCC (0.18 g, 0.88 mmol) was added and the mixture was stirred at 0°C for 30 min and the solid dicyclohexylurea was filtered off, washed with CH<sub>2</sub>Cl<sub>2</sub> and the filtrate was collected. The solution was concentrated under vacuum. The benzotriazolyl ester residue was subsequently reacted in DMF (3 mL) with D-alanine methyl ester hydrochloride salt (0.12 g, 0.93 mmol) in the presence of DIEA (0.29 mL, 1.69 mmol). The mixture was stirred overnight at *r.t.* Water (10 mL) was added to the reaction medium and the mixture was extracted with ethyl acetate (3 × 40 mL). Then the organic layer was washed with concentrated Na<sub>2</sub>CO<sub>3</sub>, 1 M KHSO<sub>4</sub>, brine, and dried over Na<sub>2</sub>SO<sub>4</sub>, concentrated under reduced pressure. The methyl ester was saponified in 0.8 M CaCl<sub>2</sub> in *i*PrOH/H<sub>2</sub>O 70:30 (10 mL) [S1]. NaOH

(1.5 eq) was added to the solution and the mixture was stirred overnight at *r.t.* The mixture was concentrated under reduced pressure, acidified with acetic acid (2 eq.) and extracted with ethyl acetate (2 × 40 ml). The organic layer was washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub> and concentrated to give the Boc-protected dipeptide as a solid residue. The Boc protecting group was removed from the solid residue (310.6 mg) by treatment with TFA/H<sub>2</sub>O (90:10 v/v) (10 mL) for 30 min. The solution was concentrated under vacuum. The oily product was treated with 15 mL cold ethyl ether then it was kept in the cold overnight. The white precipitate was filtered and washed with ether and then dried (225 mg); <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ<sub>H</sub> / ppm: 8.67 (d, *J* = 7.5 Hz, 1 H), 7.15 (d, *J* = 8.6 Hz, 2 H), 6.89 (d, *J* = 8.7 Hz, 2 H), 4.24 (p, *J* = 7.3 Hz, 1 H), 4.00 (t, *J* = 6.9 Hz, 1 H), 3.73 (s, 3 H), 3.02 – 2.84 (m, 2 H), 1.17 (d, *J* = 7.2 Hz, 3 H); <sup>13</sup>C NMR (101 MHz, DMSO-*d*<sub>6</sub>) δ<sub>C</sub> / ppm: 167.83 (s), 131.09 (s), 127.06 (s), 114.12 (s), 55.47 (s), 53.61 (s), 48.07 (s), 36.84 (s), 17.64 (s); HPLC (Method A) retention time 16.5 min; HRMS (ESI positive mode): calcd for C<sub>13</sub>H<sub>19</sub>N<sub>2</sub>O<sub>4</sub><sup>+</sup> [M+H<sup>+</sup>] 267.1345; found 267.1353.

#### **Ac-Tyr(Me)-L-Ala-OH: 2-LL** <sup>[S2]</sup>

A mixture of Boc-L-Tyr(Me)-OH (0.4 g, 1.35 mmol) and HOBt (0.22 g, 1.48 mmol) was stirred in CH<sub>2</sub>Cl<sub>2</sub> (6 mL) vigorously for 5 min. Then the solution was cooled to 0°C, DCC (0.29 g, 1.42 mmol) was added and the mixture was stirred at 0°C for 30 min and the solid dicyclohexylurea was filtered off, washed with CH<sub>2</sub>Cl<sub>2</sub> and the filtrate was recovered. The solution was concentrated under vacuum. Then the crude Boc-L-Tyr(Me)-OBt ester was subsequently reacted in DMF (3 mL) with L-alanine methyl ester (0.2 g, 1.48 mmol) in the presence of DIEA (0.47 mL, 2.7 mmol). The mixture was stirred overnight at *r.t.*, then water (10 mL) was added and it was extracted with ethyl acetate (2 × 50 mL). Then the organic layer was washed with H<sub>2</sub>O, concentrated Na<sub>2</sub>CO<sub>3</sub>, 1 M KHSO<sub>4</sub>, brine, and dried over Na<sub>2</sub>SO<sub>4</sub>, concentrated under reduced pressure. The Boc protecting group was removed from the solid residue by treatment with TFA/H<sub>2</sub>O (90:10) (10 mL) for 30 min. The solution was concentrated under vacuum. The oily product was treated with 15 mL cold ethyl ether then kept at 4°C overnight. The white solid was collected by filtration, washed with ether and dried. The white solid and DIEA (1 eq) were dissolved in CH<sub>2</sub>Cl<sub>2</sub> (10 mL). Acetic anhydride (2 eq) was added and the solution was stirred for 1 h at 0°C and then overnight at *r.t.* Water (10 mL) was added to the solution, which was extracted by ethyl acetate (2 × 50 mL). The organic layer was washed with concentrated Na<sub>2</sub>CO<sub>3</sub>, 1 M KHSO<sub>4</sub>, brine, and dried by Na<sub>2</sub>SO<sub>4</sub>, then concentrated under reduced pressure. The solid residue (280 mg) was dissolved in a 0.8 M CaCl<sub>2</sub> solution in *i*PrOH/H<sub>2</sub>O 70:30 (10 mL). 1 N NaOH (1.5 eq) was added and the solution was stirred overnight at *r.t.* The mixture was concentrated under reduced pressure and treated with water and ethyl ether. The aqueous layer was recovered, acidified with

acetic acid (2 eq) and extracted with ethyl acetate. The organic layer was washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub> and concentrated to give the product as a solid residue. The solid was dissolved in water and freeze-dried twice in order to remove excess acetic acid. Yield: 141 mg. <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>), δ<sub>H</sub> / ppm: 8.35 (d, *J* = 8.3 Hz, 1 H) 8.05 (d, *J* = 8.3 Hz, 1 H) 7.25 (d, *J* = 8.3 Hz, 2 H) 6.85 (d, *J* = 8.3 Hz, 2 H) 4.50 (m, 1 H) 4.25 (m, 1 H) 3.75 (s, 3 H) 2.50-3.00 (m, 2 H), 1.75 (s, 3 H) 1.30 (d, 3 H); HPLC (Method A) retention time 16.2 min.

**Ac-Tyr(Me)-D-Ala-OH: 2-LD** <sup>[S2]</sup>

A mixture of Boc-L-Tyr(Me)-OH (0.4 g, 1.35 mmol) and HOBt (0.22 g, 1.48 mmol) was stirred in CH<sub>2</sub>Cl<sub>2</sub> (6 mL) vigorously for 5 min. Then the solution was cooled to 0°C, DCC (0.29 g, 1.42 mmol) was added and the mixture was stirred at 0°C for 30 min and the solid dicyclohexylurea was filtered off, washed with CH<sub>2</sub>Cl<sub>2</sub> and the filtrate was recovered. The solution was concentrated under vacuum. Then the Boc-L-Tyr(Me)-OBt ester was subsequently reacted in DMF (3 mL) with D-alanine methyl ester (0.2 g, 1.48 mmol) in the presence of DIEA (0.47 mL, 2.7 mmol). The mixture was stirred overnight at *r.t.*, then water (10 mL) was added and it was extracted with ethyl acetate (2 × 50 mL). Then the organic layer was washed with H<sub>2</sub>O, concentrated Na<sub>2</sub>CO<sub>3</sub>, 1 M KHSO<sub>4</sub>, brine, and dried over Na<sub>2</sub>SO<sub>4</sub>, and finally concentrated under reduced pressure. The Boc protecting group was removed from the solid residue by treatment with TFA/H<sub>2</sub>O (90:10) (10 mL) for 30 min. The solution was concentrated under vacuum, dissolved in water, brought to pH 10 with Na<sub>2</sub>CO<sub>3</sub> and rapidly extracted with ethyl acetate. The organic layer was washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub> and concentrated under reduced pressure. The white solid and DIEA (1 eq) were dissolved in CH<sub>2</sub>Cl<sub>2</sub> (10 mL). Acetic anhydride (2 eq) was added and the solution was stirred for 1 h at 0°C and then overnight at *r.t.* Water (10 mL) was added to the solution, which was extracted by ethyl acetate (2 × 50 mL). The organic layer was washed with concentrated Na<sub>2</sub>CO<sub>3</sub>, 1 M KHSO<sub>4</sub>, brine, and dried by Na<sub>2</sub>SO<sub>4</sub>, then concentrated under reduced pressure. The solid residue was recrystallized from ethyl acetate/hexane. The solid was then dissolved in a 0.8 M CaCl<sub>2</sub> solution in *i*PrOH/H<sub>2</sub>O 70:30 (10 mL). 1 N NaOH (1.5 eq) was added and the solution was stirred overnight at *r.t.* The mixture was concentrated under reduced pressure and treated with water and ethyl ether. The aqueous layer was recovered acidified with HCl (2 eq) and extracted with ethyl acetate. The organic layer was washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub> and concentrated to give the product as a solid residue. Yield: 50 mg, <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ<sub>H</sub> / ppm: 8.28 (d, *J* = 7.4 Hz, 1 H), 8.07 (d, *J* = 8.7 Hz, 1 H), 7.14 (d, *J* = 8.6 Hz, 2 H), 6.83 (t, *J* = 9.4 Hz, 2 H), 4.51 (td, *J* = 9.1, 5.1 Hz, 1 H), 4.23 – 4.13 (m, 1 H), 3.72 (d, *J* = 5.3 Hz, 3 H), 2.85 (dd, *J* = 13.6, 5.1 Hz, 1 H), 2.66 (dd, *J* = 13.6, 9.6 Hz, 1 H), 1.76 (s, 3 H), 1.19 (d, *J* = 7.2 Hz, 3 H); HPLC (Method A) retention time 19.9 min.

### ***N*-Carbamoyl-Tyr(Me)-L-Ala-OH**

NaOCN (5 mmol, 325 mg) was dissolved in a 10 mM acetic acid solution in water (100 mL). Then the free dipeptide H-Tyr(Me)-L-Ala-OH (1 mmol, 26.61 mg) was added. The reaction mixture was stirred overnight at *r.t.* The *N*-carbamoylated dipeptide product was separated from the free dipeptide using a Dowex 50 (H<sup>+</sup> form) ion exchange resin. The fraction containing the product was eluted with water, collected and freeze-dried to give a solid residue (20 mg). <sup>1</sup>H NMR (300 MHz, D<sub>2</sub>O) δ<sub>H</sub> / ppm: 7.13 (d, *J* = 7.2 Hz, 2 H), 6.91 – 6.83 (m, 2 H), 4.29 (t, *J* = 6.6 Hz, 1 H), 4.19 (q, *J* = 7.5 Hz, 1 H), 3.73 (d, *J* = 1.5 Hz, 3 H), 2.97 (dd, *J* = 13.8, 5.8 Hz, 1 H), 2.82 (dd, *J* = 13.9, 8.3 Hz, 1 H), 1.29 – 1.20 (m, 3 H); ESI-MS (positive mode): calcd for C<sub>14</sub>H<sub>20</sub>N<sub>3</sub>O<sub>5</sub><sup>+</sup> [M+H<sup>+</sup>] 310.1398, found 310.1400 and calcd for C<sub>14</sub>H<sub>20</sub>N<sub>3</sub>O<sub>5</sub><sup>+</sup> [M+Na<sup>+</sup>] 310.1217, found 332.12; HPLC analysis (method A): retention time 14.4 min.

### **Methylphosphate, di-sodium salt**

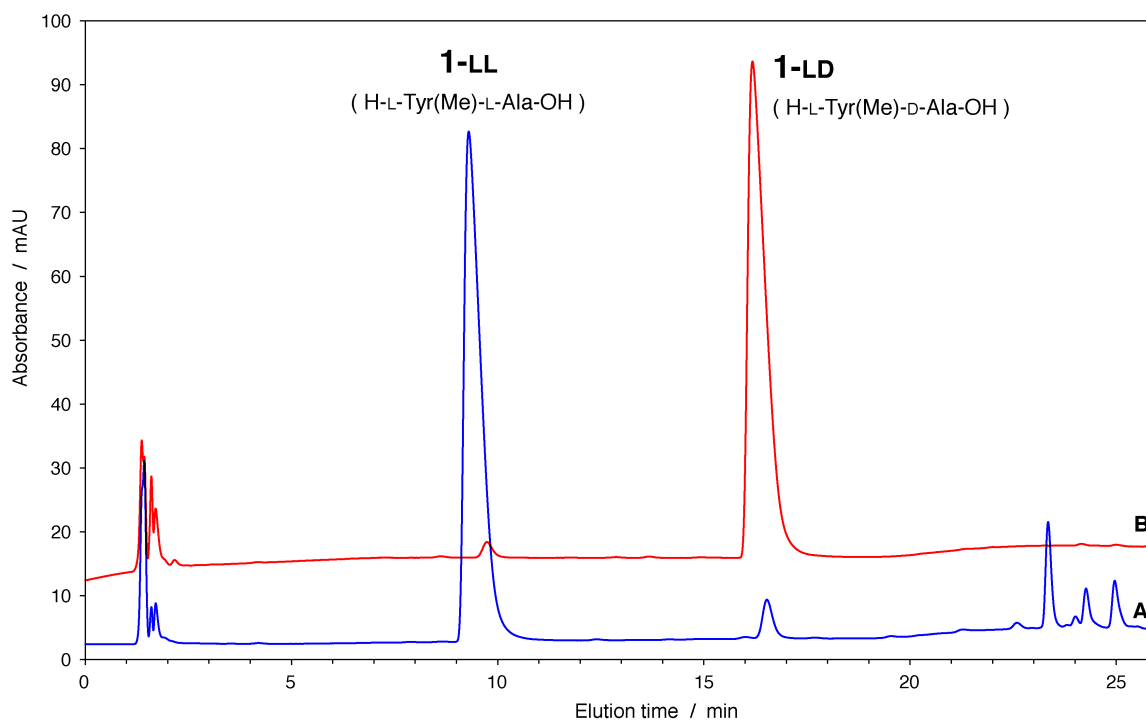
Cyclohexylamine was exchanged for Na<sup>+</sup> from the dicyclohexylammonium salt [S3] using the following procedure. Firstly the cyclohexylamine was removed from the salt (1 mmol, 0.31 g) dissolved in water (10 mL) using a Dowex 50 (H<sup>+</sup> form) ion exchange resin. The solution was introduced and the column was extensively eluted with water. The eluate was collected in a flask containing NaHCO<sub>3</sub> (2 mmol, 168 mg) and then freeze-dried to lead to the di-sodium salt. <sup>1</sup>H NMR (300 MHz, D<sub>2</sub>O) δ<sub>H</sub> / ppm: 3.35 (d, *J* = 10.1 Hz, 3 H).

### **Procedures and results of activation experiments**

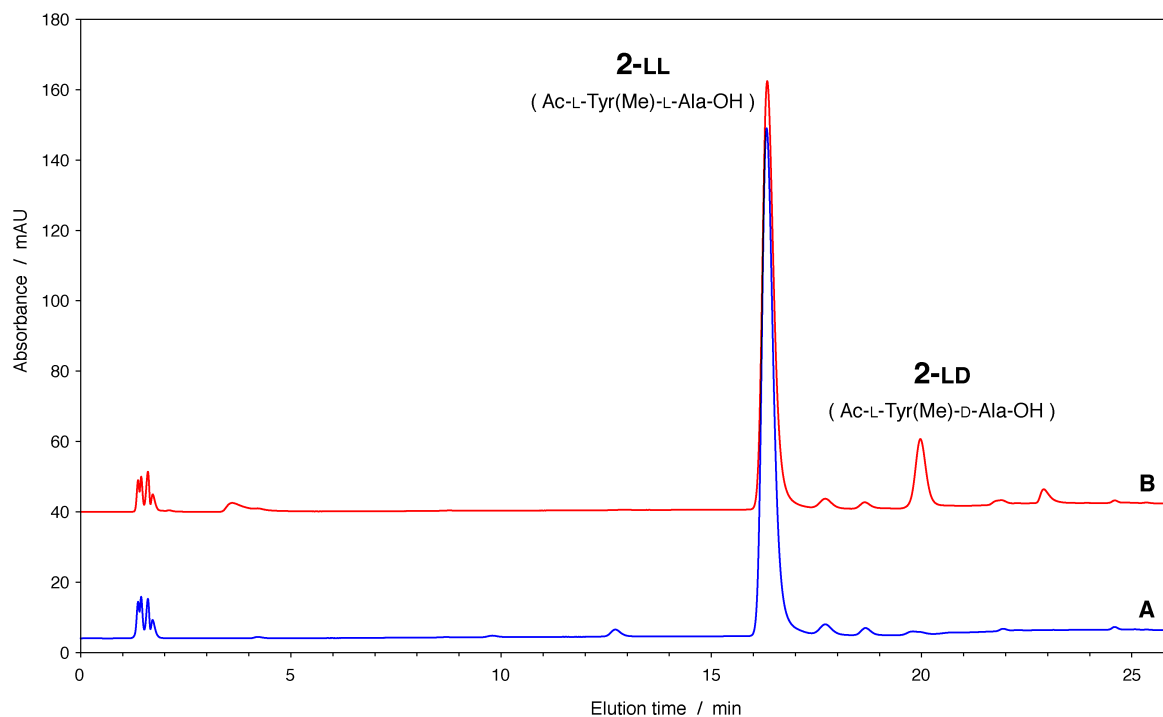
Buffers were prepared according to the following procedures: 100 mM MES buffers pH 5.5 and 6.5 were prepared on a 100 mL scale by dissolving MES (2-morpholinoethanesulfonic acid, monohydrate) (2.1 g, 10 mmol) in water and adding 1 N NaOH (either 1.8 mL for pH 5.5 or 6.9 mL for pH 6.5) in a volumetric flask (100 mL). 50 mM methyl phosphate buffer at pH 7.1 was prepared by dissolving di-sodium methyl phosphate (0.117 g, 0.75 mmol) salt in water (15 ml), then the pH was adjusted to pH 7.1 by a few drops of 0.2 M HCl.

## EDC-promoted epimerization of dipeptides H-Tyr(Me)-L-Ala-OH (**1-LL**) and Ac-Tyr(Me)-L-Ala-OH (**2-LL**)

This reaction was performed according to a protocol similar to that used earlier [S2] for several dipeptides. The dipeptides **1-LL** or **2-LL** (0.01 mmol) were dissolved in 100 mM MES buffer (pH 6.5, 10 mL), the solution was stirred at *r.t.* and solid EDC (1 eq, 1.9 mg) was added to the solution. The epimerization was monitored by HPLC (method A). The result are reported in figures S1 and S2 and Table 1 entries 1 and 2.



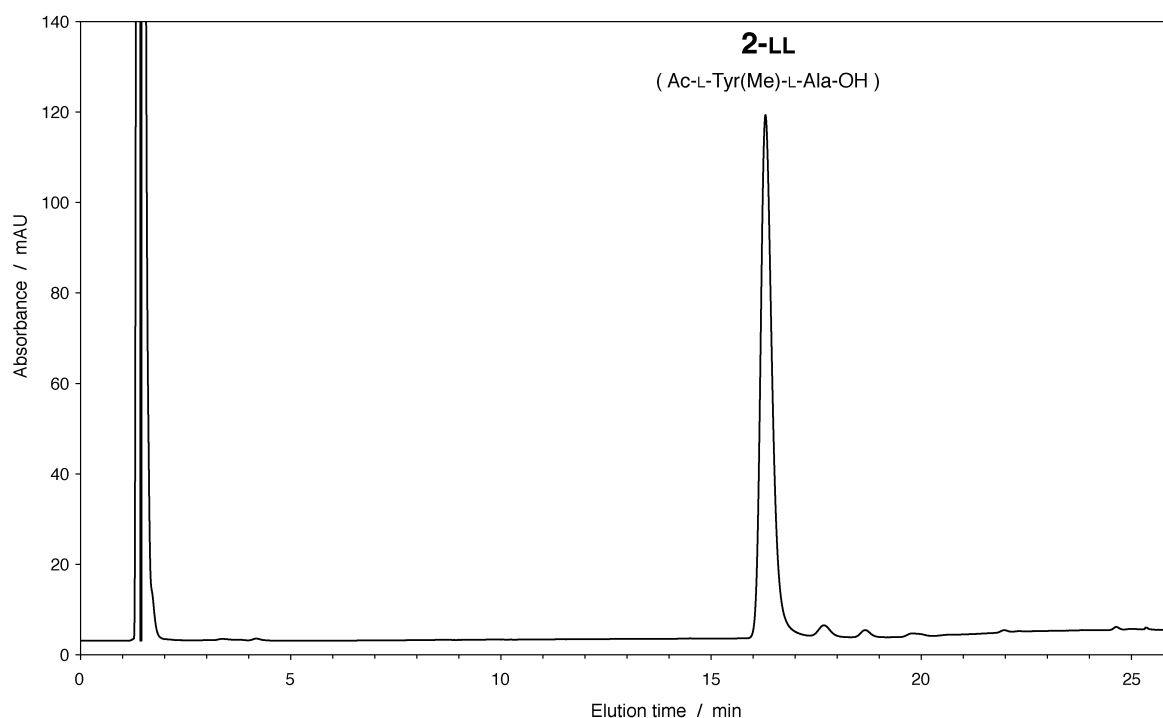
**Figure S1** Reaction of 1 mM H-Tyr(Me)-L-Ala-OH dipeptide **1-LL** with EDC (1 eq., 1 mM) in 100 mM MES buffer (pH 6.5) at room temperature. HPLC chromatogram (method A, UV detection 273 nm). **A**: Reaction mixture after 4 days. **B**: authentic sample of the stereoisomer **1-LD** (H-Tyr(Me)-D-Ala-OH).



**Figure S2** Reaction of 1 mM Ac-Tyr(Me)-L-Ala-OH dipeptide **2-LL** with EDC (1 eq., 1 mM) in 100 mM MES buffer (pH 6.5). HPLC chromatogram (method A, UV detection 273 nm). **A**: Solution of the starting material **2-LL**. **B**: reaction medium after 3 days at room temperature.

**Reaction of 1 mM Ac-Tyr(Me)-L-Ala-OH 2-LL in presence of 5 mM CuSO<sub>4</sub> and 10 mM KCN in MES buffer:**

A 1 mM Ac-Tyr(Me)-L-Ala-OH **2-LL** stock solution was prepared by dissolving (0.01 mmol, 3.08 mg) in 100 mM MES buffer (pH 6.5, 10 mL). A 2 mL sample of the preceding solution was added to solid CuSO<sub>4</sub> (0.01 mmol, 1.6 mg) and KCN (0.02 mmol, 1.3 mg). The mixture was stirred until complete dissolution of the solids and kept at room temperature. The progress of the reaction of *in situ* generated cyanogen was monitored by HPLC (Method A) during 18 days (Figure S3, Table 1 entry 3).

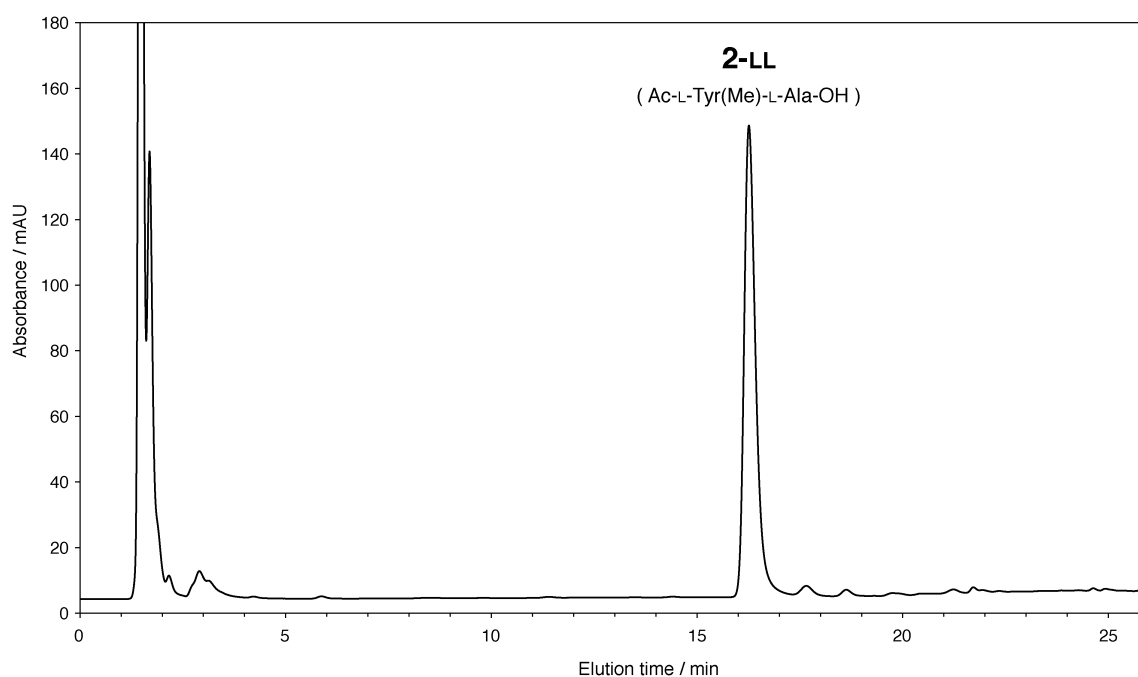


**Figure S3** Reaction of 1 mM Ac-Tyr(Me)-L-Ala-OH dipeptide **2-LL** with a mixture of 5 mM CuSO<sub>4</sub> and 10 mM KCN in 100 mM MES buffer (pH 6.5). HPLC chromatograms (method A, UV detection 273 nm) of the reaction medium after 18 days at room temperature.



**Reaction of 1 mM Ac-Tyr(Me)-L-Ala-OH 2-LL in presence of a mixture of CuSO<sub>4</sub> and KCN (5 mM, 10 mM) in 50 mM methyl phosphate buffer:**

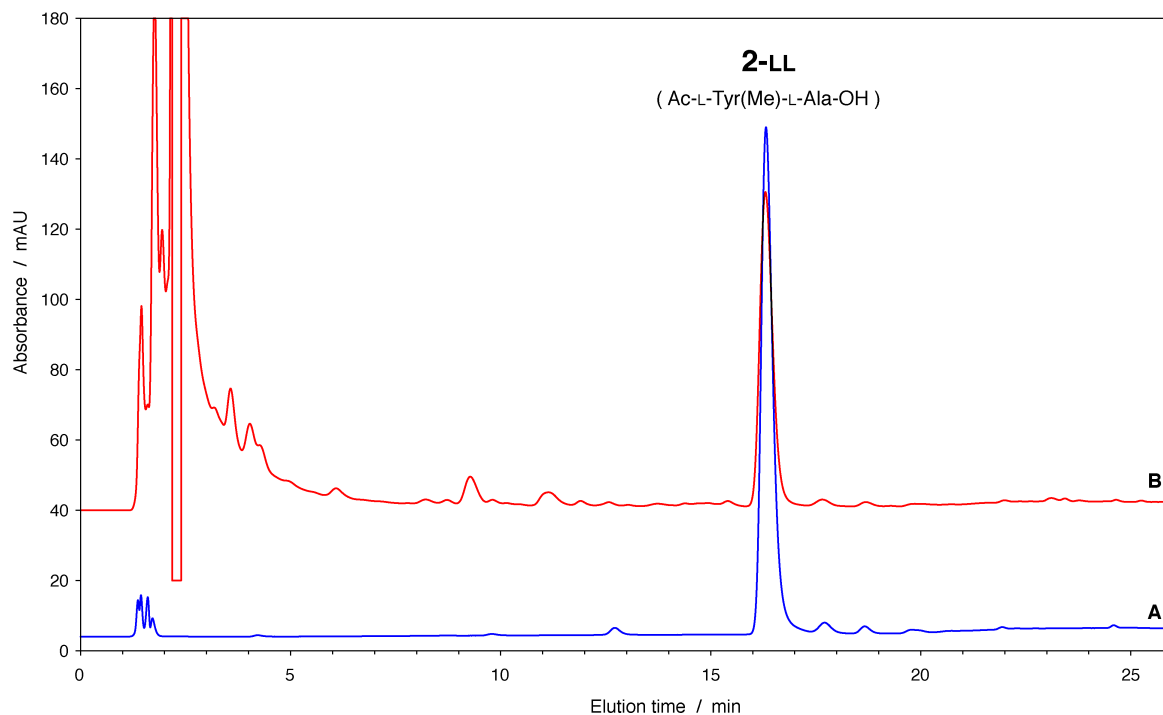
A 1 mM Ac-Tyr(Me)-L-Ala-OH 2-LL stock solution was prepared by dissolving (0.01 mmol, 3.08 mg) in 50 mM methyl phosphate buffer (pH 7.1, 10 mL). A 2 mL sample of the previous stock solution was added to a mixture of solid CuSO<sub>4</sub> (0.01, 1.6 mg) and KCN (0.02 mmol, 1.3 mg). The reaction was kept at room temperature and the reaction progress was monitored by HPLC (Method A) during 4 days (Figure S4, Table 1 entry 4).



**Figure S4** Reaction of 1 mM Ac-Tyr(Me)-L-Ala-OH dipeptide 2-LL with a mixture of 5 mM CuSO<sub>4</sub> and 10 mM KCN in 50 mM methyl phosphate (pH 7.1). HPLC chromatograms (method A, UV detection 273 nm) of the reaction medium after 4 days at room temperature.

**Reaction of 1 mM Ac-Tyr(Me)-L-Ala-OH 2-LL with 10 mM DAMN at room temperature:**

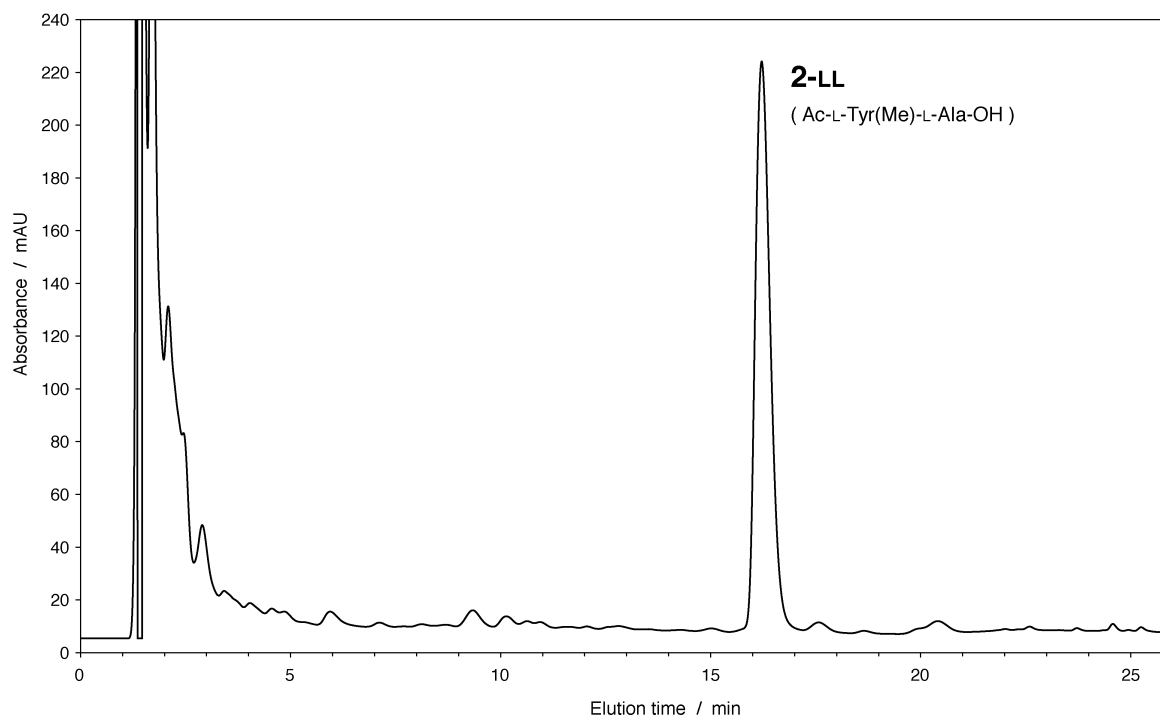
A 1 mM Ac-Tyr(Me)-L-Ala-OH 2-LL stock solution was prepared by dissolving (0.01 mmol, 3.08 mg) in 100 mM MES buffer (pH 6.5, 10 mL). A 3 mL sample was taken from the above stock solution and added to (0.03 mmol, 3.2 mg) DAMN. The solution was kept at room temperature and the reaction progress was monitored by HPLC (Method A) during 11 days (Figure S5, Table 1 entry 5).



**Figure S5** Reaction of 1 mM Ac-Tyr(Me)-L-Ala-OH dipeptide 2-LL with 10 mM DAMN in 100 mM MES buffer (pH 6.5). HPLC chromatograms (method A, UV detection 273 nm). **A**: Solution of the starting material 2-LL. **B**: Reaction medium after 11 days at room temperature.

**Reaction of 10 mM Ac-Tyr(Me)-L-Ala-OH **2-LL** with 20 mM DAMN at 90 °C:**

A 1 mM Ac-Tyr(Me)-L-Ala-OH **2-LL** solution was prepared by dissolving (0.01 mmol, 3.08 mg) in 100 mM MES buffer (pH 6.5, 10 mL). Then the solution was added to HCN-tetramer (0.2 mmol, 21.6 mg). The solution was kept under stirring at 90 °C and the reaction progress was monitored by HPLC (Method A) during 25 days (Figure S6, Table 1 entry 6).



**Figure S6** Reaction of 1 mM Ac-Tyr(Me)-L-Ala-OH dipeptide **2-LL** with 20 mM HCN-tetramer in 100 mM MES buffer (pH 6.5). HPLC chromatogram (method A, UV detection 273 nm) of the reaction medium after 25 days at 90 °C.

**Study of reactivity of 1 mM Ac-Tyr(Me)-L-Ala-OH 2-LL with 40 mM cyanamide in MES buffer:**

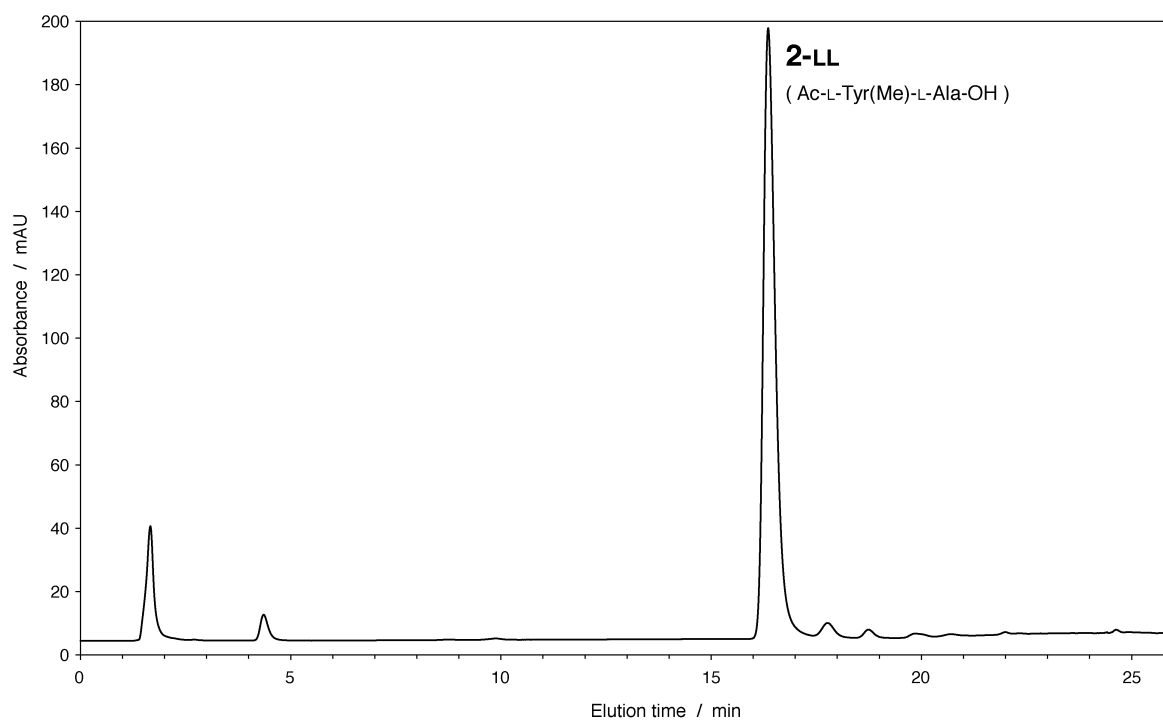
A 1 mM Ac-Tyr(Me)-L-Ala-OH **2-LL** solution was prepared by dissolving (0.01 mmol, 3.08 mg) dipeptide in 100 mM MES buffer (pH 5.5, 10 mL). Then the previous solution was added to a round bottom flask containing cyanamide (0.4 mmol, 16.8 mg). The solution was kept under stirring at 80 °C and the reaction progress was monitored by HPLC (Method A) during 20 days (Figure 1 main document, Table 1 entry 7, Table S1).

**Table S1** Evolution of the area % of Ac-Tyr(Me)-L-Ala-OH **2-LL** and Ac-Tyr(Me)-D-Ala-OH **2-LD** in the reaction medium of 1 mM **2-LL** Ac-Tyr(Me)-L-Ala-OH with (40 mM) cyanamide in MES buffer (pH 5.5) at 80 °C during 20 days. The hydrolysis of the dipeptides was considered to account for the difference of the sum to 100%.

<i>t</i> / days	<b>2-LL</b>	<b>2-LD</b>
0	96.4	0.7
2	95.7	1.3
8	93.6	2.1
20	88.5	5.4

**Reaction of 1 mM Ac-Tyr(Me)-L-Ala-OH 2-LL with 10 mM cyanamide in 5 mM imidazole buffer:**

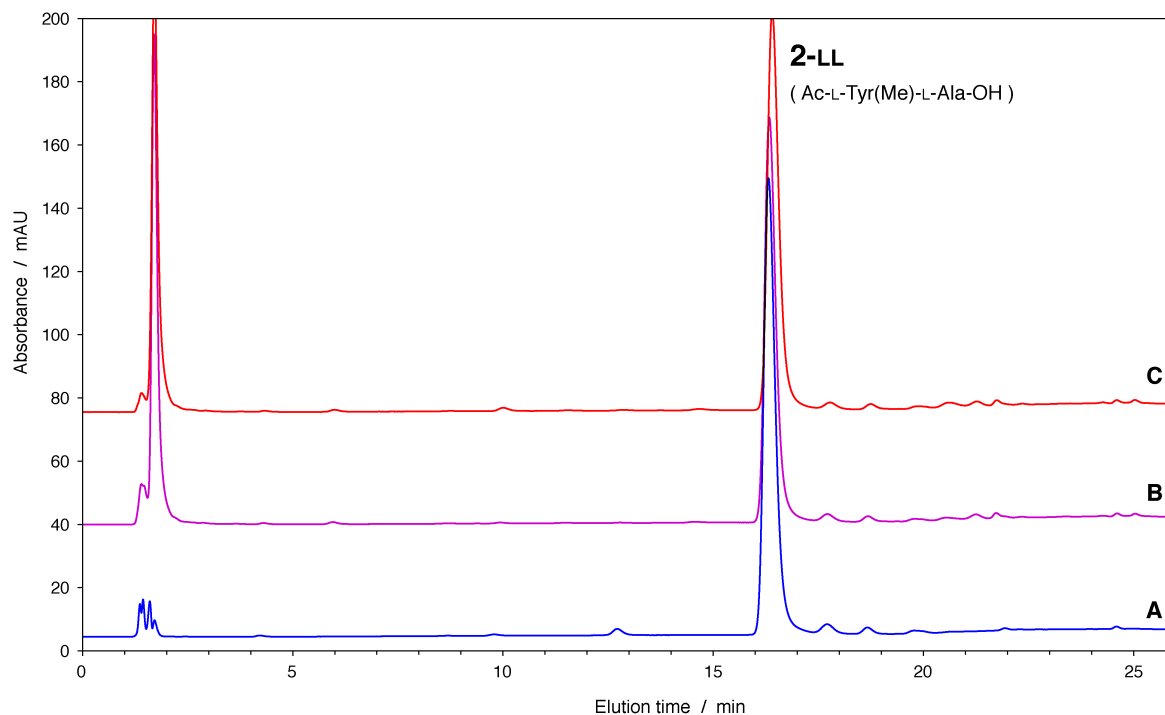
Solid cyanamide (0.04 mmol, 1.7 mg) was dissolved in 4 mL of 5 mM imidazole buffer solution (pH 6.68). Then the previous solution was added subsequently to dipeptide **2-LL** (4  $\mu$ mol, 1.23 mg). The solution was kept at room temperature and the reaction progress was monitored by HPLC (Method A) during 7 days (Figure S7, Table 1 entry 8).



**Figure S7** Reaction of 1 mM Ac-Tyr(Me)-L-Ala-OH dipeptide **2-LL** with 10 mM cyanamide in 5 mM imidazole buffer (pH 6.68). HPLC chromatogram (method A, UV detection 273 nm) of the reaction medium after 7 days

**Study of the reactivity of 1 mM Ac-Tyr(Me)-L-Ala-OH 2-LL with 10 mM cyanamide in 50 mM methyl phosphate buffer:**

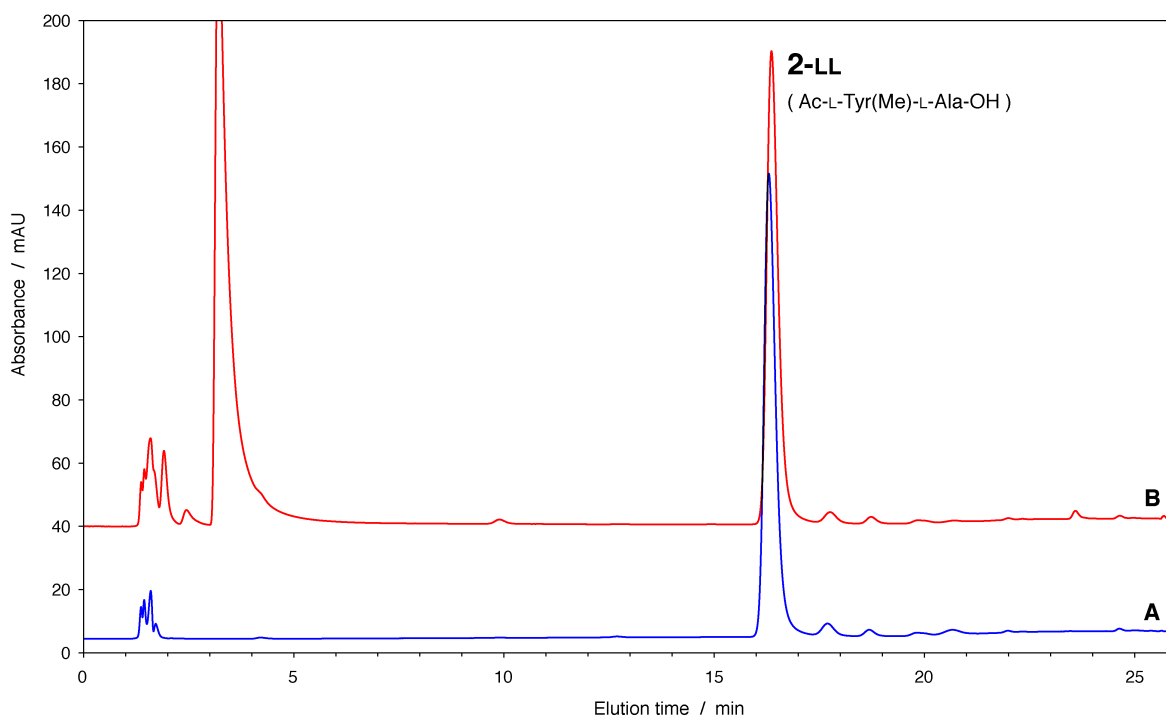
Solid cyanamide (0.04 mmol, 1.7 mg) was dissolved in 4 mL of 50 mM methyl phosphate buffer solution (pH 7.1). This solution was subsequently added to the dipeptide **2-LL** (4  $\mu$ mol, 1.23 mg). The solution was kept at room temperature and the reaction progress was monitored by HPLC (Method A) during 13 days (Figure S8, Table 1 entry 9).



**Figure S8** Reaction of 1 mM Ac-Tyr(Me)-L-Ala-OH dipeptide **2-LL** with 10 mM cyanamide in 50 mM sodium methyl phosphate buffer (pH 7.1). HPLC chromatograms (method A, UV detection 273 nm), **A**: authentic sample Ac-Tyr(Me)-L-Ala-OH in H<sub>2</sub>O; **B**: reaction medium after 2 days at room temperature; **C**: reaction medium after 13 days.

**Reaction of 1 mM Ac-Tyr(Me)-L-Ala-OH 2-LL with 15 mM cyanamide in the presence of 1 mM CuCl<sub>2</sub> in MES buffer:**

A 1 mM Ac-Tyr(Me)-L-Ala-OH stock solution was prepared by dissolving **2-LL** (0.01 mmol, 3.08 mg) in 100 mM MES buffer (pH 6.5, 10 mL). A 2 mL sample from the above stock solution was added to a mixture of solid cyanamide (0.03 mmol, 1.26 mg) and CuCl<sub>2</sub> (0.002 mmol, 0.27 mg). The solution was kept at room temperature and the reaction progress was monitored by HPLC (Method A) during 5 days (Figure S9, Table 1 entry 10).



**Figure S9** Reaction of 1 mM Ac-Tyr(Me)-L-Ala-OH dipeptide **2-LL** with 15 mM cyanamide in the presence of 1 mM CuCl<sub>2</sub> in MES buffer (pH 6.5). HPLC chromatograms (method A, UV detection 273 nm), **A**: solution of the starting material; **B**: reaction medium after 5 days.

### Reaction of 1 mM Ac-Tyr(Me)-L-Ala-OH 2-LL with 5 mM cyanamide in the presence or absence of 5 mM glyoxylic acid in MES buffer

Three stock solutions were prepared.

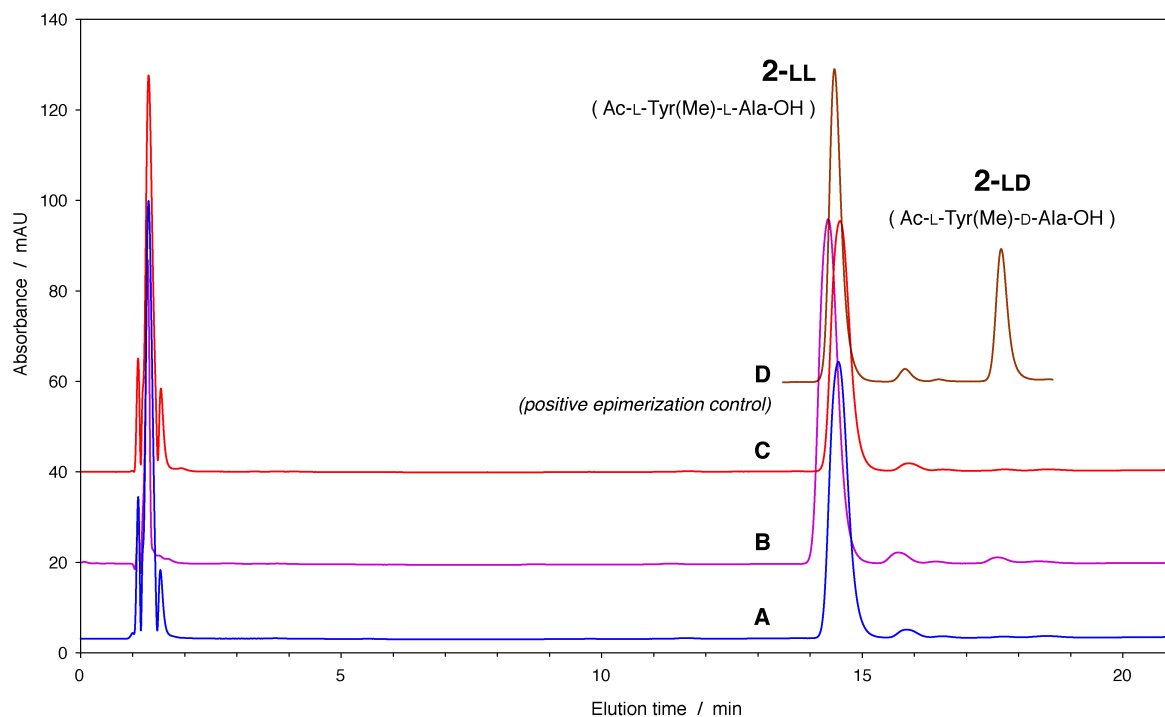
- A. Solution A (12 mM dipeptide 2-LL stock solution): (18.48 mg) in 100 mM MES buffer (pH 5, 5 mL).
- B. Solution B (41 mM cyanamide stock solution): (33.03 mg) in 100 mM MES buffer (pH 5, 10 mL).
- C. Solution C (100 mM glyoxylic acid stock solution): (91.60 mg) in 100 mM MES buffer (pH 5, 10 mL).

Then four experiments (1, 2, 3, 4) were carried out from mixtures of the stock solutions prepared according to the table S2. The volume of each sample was completed to 2 ml with 100 mM MES buffer pH 5. The samples (1, 2, 3) were kept at 40 °C, and the progress of the reactions was monitored by HPLC (Method B) during 5 days (Figure S10, Table 1 entry 11).

**Table S2** Composition of the solutions used for the analysis of the reaction of 1 mM Ac-Tyr(Me)-L-Ala-OH 2-LL with 5 mM cyanamide in the presence or absence of 5 mM glyoxylic acid. Experiments 1–2 constitute negative controls and experiment 4 is added as a positive control for epimerization in the presence of EDC.

Exp.	A 2-LL	B Cyanamide	C Glyoxylate	MES Buffer pH 5	EDC
1	0.17 mL	-	-	1.83 mL	0
2	0.17 mL	0.25 mL	-	1.58 mL	0
3	0.17 mL	0.25 mL	0.1 mL	1.48 mL	0
4 (control)	0.17 mL	-	-	1.83 mL	0.38 mg

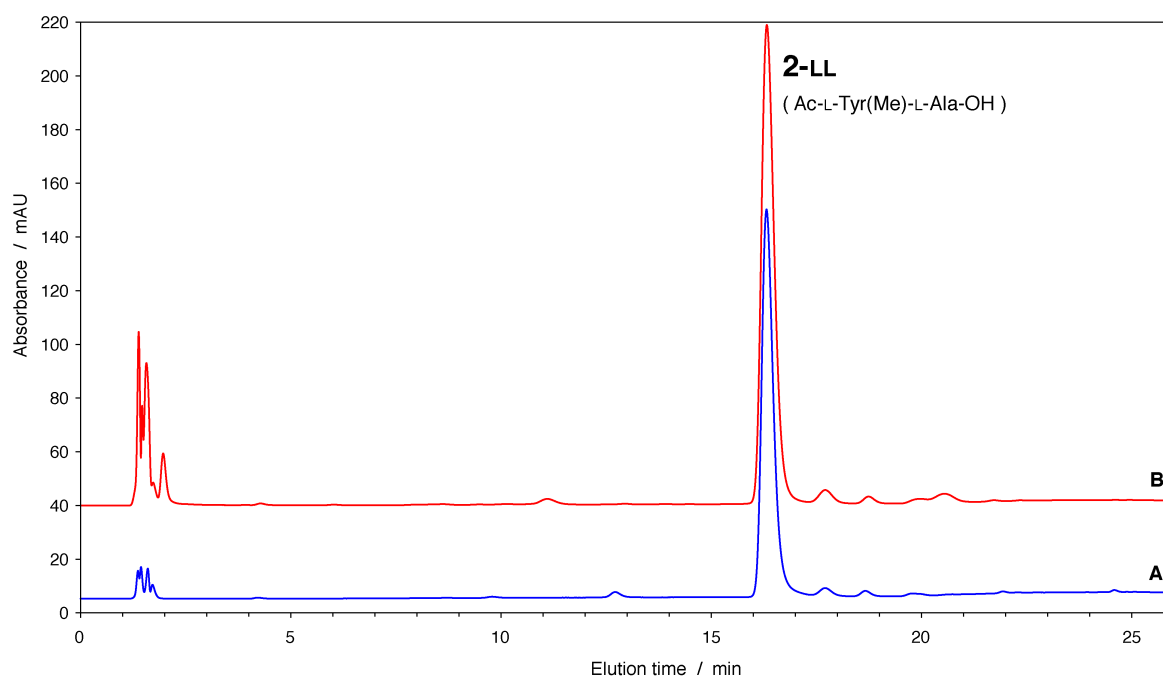




**Figure S10** Reaction of 1 mM Ac-Tyr(Me)-L-Ala-OH dipeptide **2-LL** with 5.1 mM cyanamide and 5 mM glyoxylic acid in 100 mM MES buffer (pH 5). **A**: HPLC chromatograms (method B, UV detection 273 nm) of a sample of the starting material; **B**: reaction medium in the absence of glyoxylic acid after 5 days at 40 °C; **C**: reaction medium in the presence of 5 mM glyoxylic acid after 5 days at 40 °C; **D**: reaction medium of 1 mM Ac-Tyr(Me)-L-Ala-OH 1 eq. of EDC in 100 mM MES buffer (pH 5) after 1 day of reaction (positive control for **2-LD**).

### Reaction of 1 mM Ac-Tyr(Me)-L-Ala-OH 2-LL in the presence of 20 mM urea:

A 1 mM Ac-Tyr(Me)-L-Ala-OH 2-LL solution was prepared by dissolving (0.01 mmol, 3.08 mg) in 100 mM MES buffer (pH 5.5, 10 mL). Then it was added to a round bottom flask containing urea (0.2 mmol, 12 mg). The solution was kept under stirring at 80 °C and the reaction progress was monitored by HPLC (Method A) during 20 days (Figure S11, Table 1 entry 12).



**Figure S11** Reaction of 1 mM Ac-Tyr(Me)-L-Ala-OH dipeptide 2-LL with 20 mM urea in 100 mM MES buffer (pH 5.5). HPLC chromatograms (method A, UV detection 273 nm) of **A**: a solution of the starting material; **B**: the reaction medium after 20 days at 80 °C.

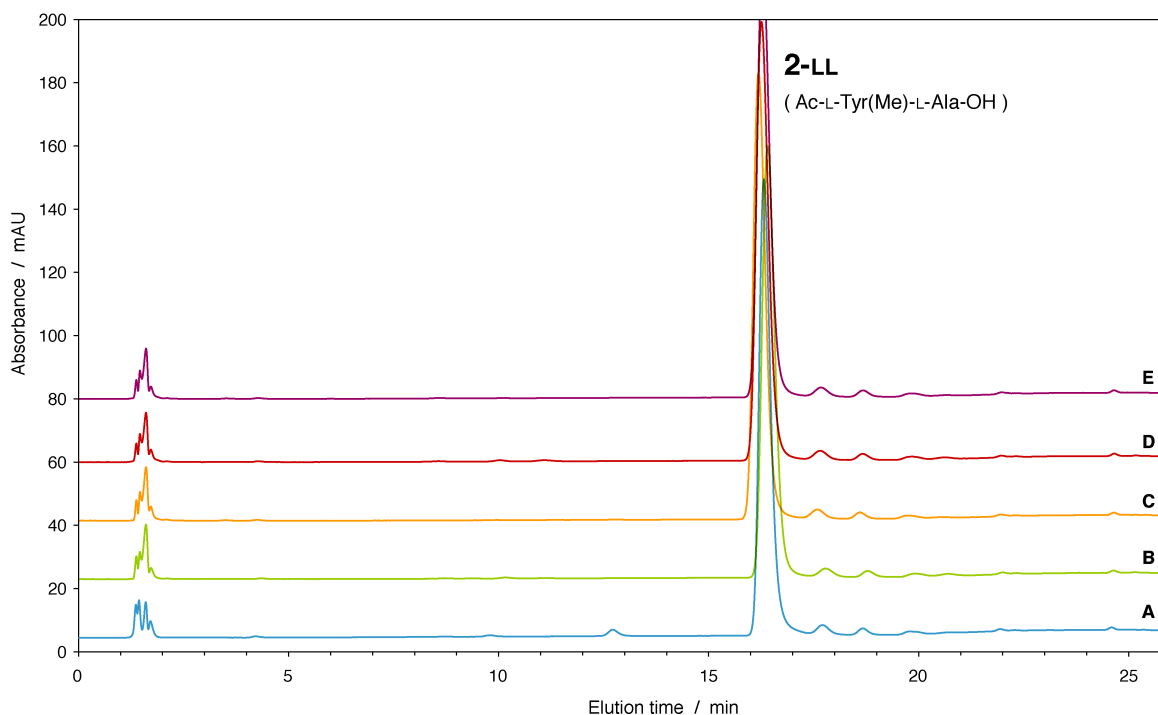
### Reaction of 1 mM Ac-Tyr(Me)-L-Ala-OH 2-LL with 5 mM sodium dicyanamide in the absence or in the presence of transition metals ( $\text{Cu}^{2+}$ , $\text{Ni}^{2+}$ , $\text{Mn}^{2+}$ ) in MES buffer at *r.t.*

A 1 mM Ac-Tyr(Me)-L-Ala-OH stock solution was prepared by dissolving (0.01 mmol, 3.08 mg) in 100 mM MES buffer (pH 6.5, 10 mL). Then 4 reactions were carried out as follows.

- **Reaction 1**: a 2 mL sample was taken from the above stock solution and it was added to (0.01 mmol, 0.94 mg) sodium dicyanamide.

- **Reaction 2:** 2 mL sample was taken from the above stock solution and it was added to a solid mixture of sodium dicyanamide (0.01 mmol, 0.94 mg) and  $\text{Cu}(\text{OAc})_2$  (0.01 mmol, 1.8 mg).
- **Reaction 3:** 2 mL sample was taken from the above stock solution and it was added to a solid mixture of sodium dicyanamide (0.01 mmol, 0.94 mg) and  $\text{NiSO}_4$  (0.01 mmol, 1.54 mg).
- **Reaction 4:** 2 mL sample was taken from the above stock solution and it was added to a solid mixture of sodium dicyanamide (0.01 mmol, 0.94 mg) and  $\text{MnSO}_4$  (0.01 mmol, 1.5 mg).

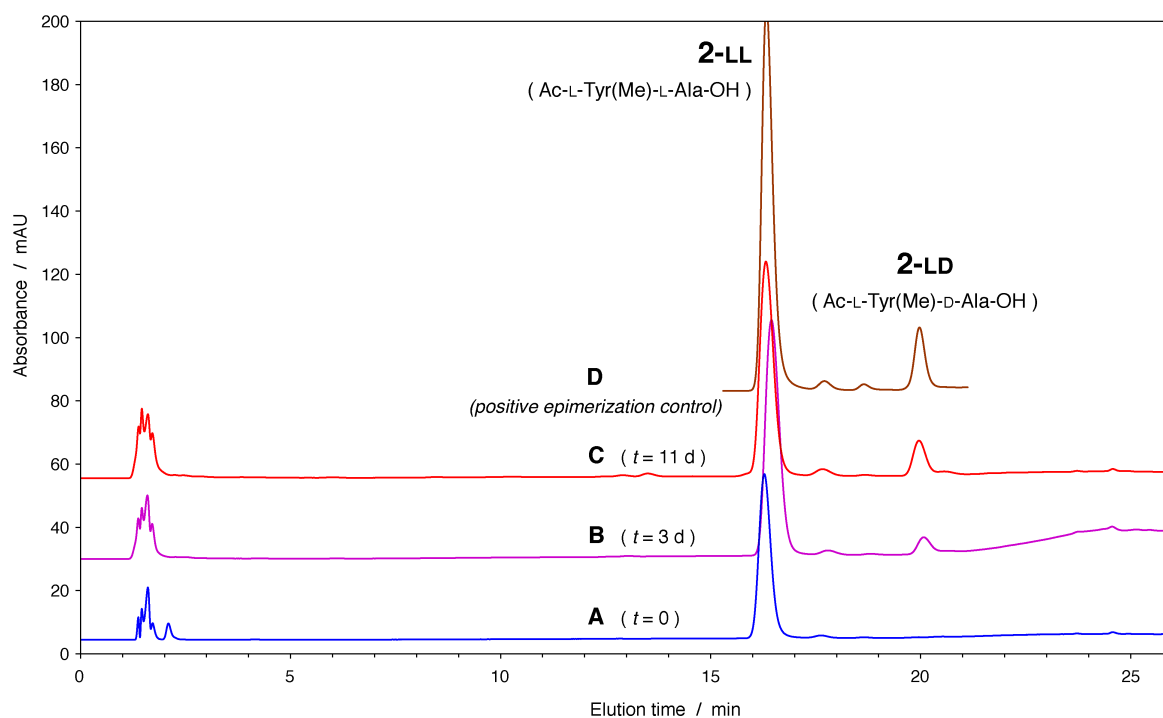
The reactions were allowed to stand at *r.t.*, and the progress of was monitored by HPLC (Method A) during 6 days (Figure S12, Table 1 entries 13–16).



**Figure S12** Reaction of 1 mM Ac-Tyr(Me)-L-Ala-OH dipeptide **2-LL** with 5 mM sodium dicyanamide in a 100 mM MES buffer (pH 5.5). HPLC chromatogram (method A, UV detection 273 nm) of **A**: the starting material; **B**: Reaction medium after 6 days at room temperature; **C**: reaction carried out in the presence of 5 mM  $\text{Cu}(\text{OAc})_2$ ; **D**: reaction carried out in the presence of 5 mM  $\text{NiSO}_4$ ; **E**: reaction carried out in the presence of 5 mM  $\text{MnSO}_4$ .

## Reaction of 1 mM Ac-Tyr(Me)-L-Ala-OH 2-LL with 5 mM sodium dicyanamide in MES buffer at 85 °C

1 mM Ac-Tyr(Me)-L-Ala-OH solution was prepared by dissolving (0.002 mmol, 0.616 mg) dipeptide in 100 mM MES buffer (pH 5.5, 2 mL). Then the previous solution was added to sodium dicyanamide (0.01 mmol, 0.94 mg). The solution was kept under stirring at 85 °C and the reaction progress was monitored by HPLC (Method A) during 11 days (Figure S13, Table 1 entry 17, Table S3).



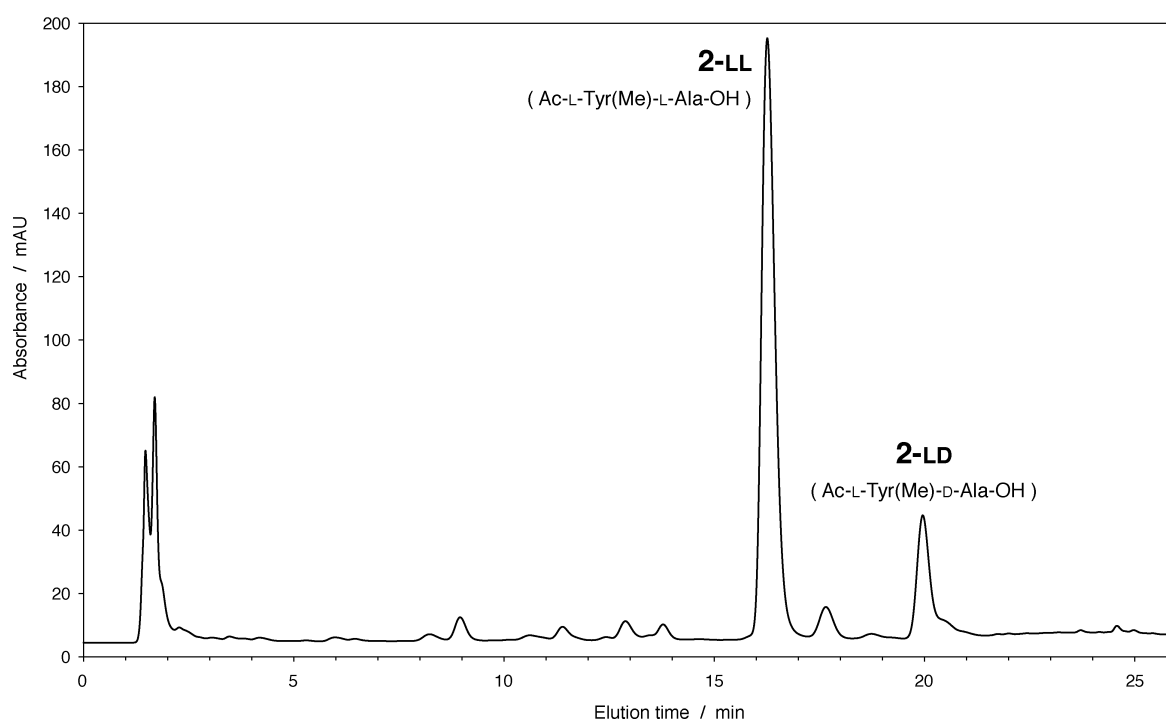
**Figure S13** Reaction of 1 mM Ac-Tyr(Me)-L-Ala-OH dipeptide **2-LL** with 5 mM sodium dicyanamide in 100 mM MES buffer (pH 5.5). **A:** HPLC chromatograms (method A, UV detection 273 nm) of the starting material; **B:** reaction medium after 3 days at 85°C; **C:** reaction medium after 11 days at 85°C; **D:** Reaction of 1 mM **2-LL** promoted by 1 eq. of EDC in 100 mM MES buffer pH 6.5 at after 3 days at room temperature (positive control for **2-LD**).

**Table S3.** Evolution of the relative area % of Ac-Tyr(Me)-L-Ala-OH **2-LL** (HPLC retention time 16.28 min) and Ac-Tyr(Me)-D-Ala-OH **2-LD** (HPLC retention time 19.95 min) in the reaction medium of 1 mM Ac-Tyr(Me)-Ala-OH with 5 mM dicyanamide in MES buffer (pH 5.5) at 85 °C during 11 days.

Reaction time	<b>2-LL</b>	<b>2-LD</b>
0	97.4%	0.1%
3 days	92.5%	5.2%
5 days	90.6%	7.7%
11 days	84.6%	12.8%

### Reaction of 10 mM Ac-Tyr(Me)-L-Ala-OH **2-LL** with 30 mM sodium dicyanamide in MES buffer at 85 °C

10 mM Ac-Tyr(Me)-L-Ala-OH **2-LL** was prepared by dissolving (0.1 mmol, 30.8 mg) dipeptide 100 mM MES buffer (pH 5.5, 10 mL). Then the previous solution was added to a (0.3 mmol, 26.7 mg) sodium dicyanamide. The solution was kept under stirring at 85 °C and the reaction progress was monitored by HPLC (Method A) for 3 days (Figure S14, Table 1 entry 18). The presence acetylated dipeptides **2-LL** and **2-LD** was confirmed by HPLC-MS (positive mode  $m/z$  309.15  $[M+H]^+$ , 331.13  $[M+Na]^+$ ) (Figure S14, Table 1 entry 18, Table S4).



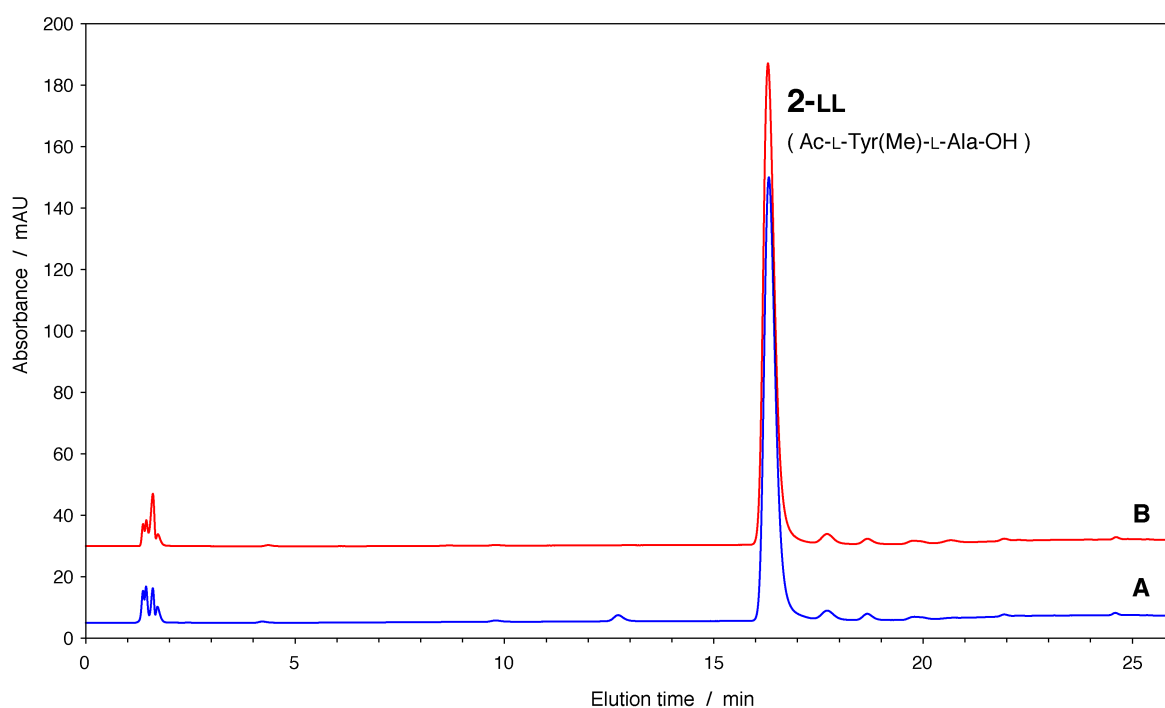
**Figure S14** Reaction of 10 mM Ac-Tyr(Me)-L-Ala-OH dipeptide **2-LL** with 30 mM dicyanamide in 100 mM MES buffer (pH 5.5). HPLC chromatograms (method A, UV detection 273 nm) of the reaction medium after 3 days at 85 °C.

**Table S4.** Evolution of the relative area % of dipeptides **2-LL** (HPLC retention time 16.28 min) and **2-LD** (HPLC retention time 19.95 min) in the reaction of 1 mM Ac-Tyr(Me)-Ala-OH **2-LL** with 30 mM dicyanamide in MES buffer (pH 5.5) at 85 °C over 3 days.

Reaction time	<b>2-LL</b>	<b>2-LD</b>
0	97.4%	0.1%
3 days	78,3%	15,5%

**Reaction of 1 mM Ac-Tyr(Me)-L-Ala-OH 2-LL with 10 mM dicyandiamide (DCDA) at *r.t.***

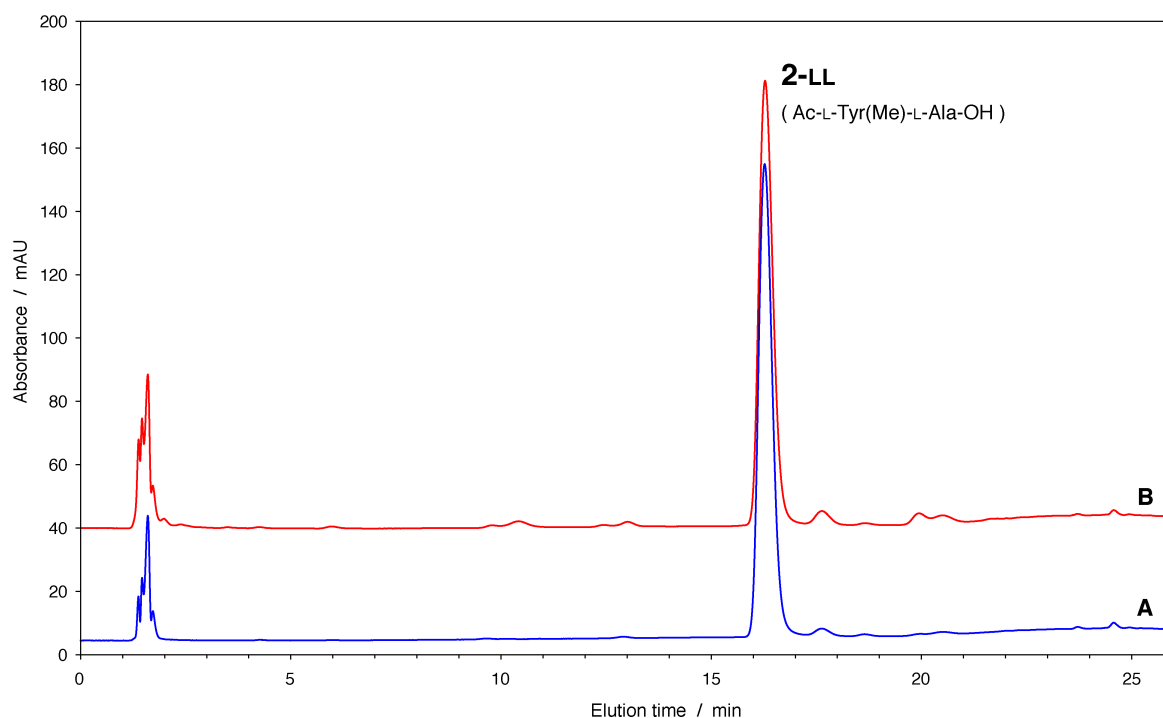
A 1 mM dipeptide **2-LL** solution was prepared by dissolving (0.002 mmol, 0.616 mg) dipeptide in 100 mM MES buffer (pH 5.5, 2 mL). Then the previous solution was added to dicyandiamide (0.02 mmol, 1.68 mg). The solution was kept at room temperature in the dark and the reaction progress was monitored by HPLC (Method A) during 3 days (Figure S15, Table 1 entry 19).



**Figure S15** Reaction of 1 mM Ac-Tyr(Me)-L-Ala-OH dipeptide **2-LL** with 10 mM dicyandiamide in 100 mM MES buffer (pH 5.5). HPLC chromatograms (method A, UV detection 273 nm) of **A**: a solution of the starting material; **B**: the reaction medium after 3 days in dark at *r.t.*

## Reaction of 1 mM Ac-Tyr(Me)-L-Ala-OH with 10 mM dicyandiamide (DCDA) at 80 °C

A 1 mM dipeptide **2-LL** solution was prepared by dissolving (0.01 mmol, 3.08 mg) dipeptide in 100 mM MES buffer (pH 5.5, 10 mL). Then the previous solution was added to dicyandiamide (0.1 mmol, 8.4 mg). The solution was kept under stirring at 80 °C in dark and the reaction progress was monitored by HPLC (Method A) during 22 days (Figure S16, Table 1 entry 20, Table S5).



**Figure S16** Reaction of 1 mM Ac-Tyr(Me)-L-Ala-OH dipeptide **2-LL** with 10 mM dicyandiamide in 100 mM MES buffer (pH 5.5). **A**: HPLC chromatograms (method A, UV detection 273 nm) of the reaction medium after one day in the dark at 80 °C; **B**: reaction medium after 22 days.

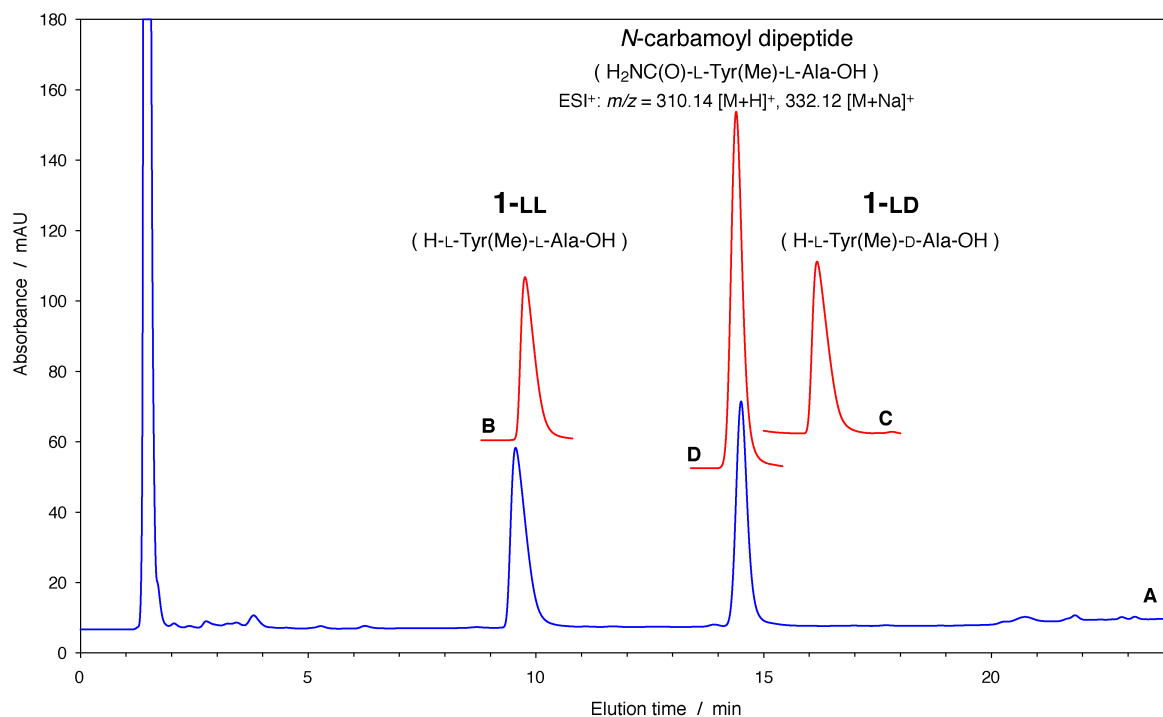
**Table S5.** Evolution of the relative area % of Ac-Tyr(Me)-L-Ala-OH (HPLC retention time 16.28 min) and Ac-Tyr(Me)-D-Ala-OH (HPLC retention time 19.95 min) in the reaction medium of 1 mM Ac-Tyr(Me)-Ala-OH by (10 mM) dicyandiamide in MES buffer (pH 5.5) at 80 °C in the dark during 22 days.

Reaction time	<b>2-LL</b>	<b>2-LD</b>
0	97.4%	0.1%
7 days	96.3%	0.95%
22 days	93.9%	2.1%



### Reaction of 1 mM H-Tyr(Me)-L-Ala-OH 1-LL in the presence of a mixture of 5 mM CuSO<sub>4</sub> and 10 mM KCN in MES

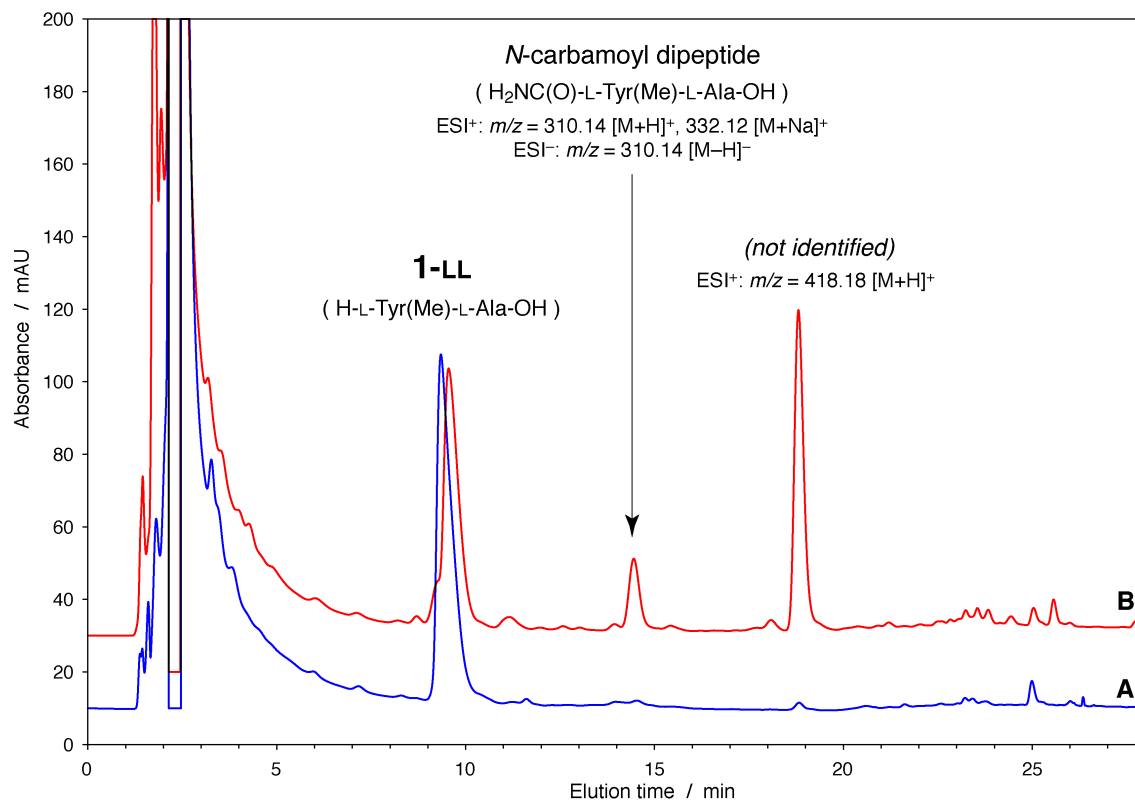
A 1 mM H-Tyr(Me)-L-Ala-OH 1-LL stock solution was prepared by dissolving 0.01 mmol, 3.08 mg) dipeptide 1-LL in 100 mM MES buffer (10 mL, pH 6.5). A sample of the above stock solution (2 mL) then was added to a mixture of solid CuSO<sub>4</sub> (10<sup>-2</sup> mmol, 1.6 mg) and KCN (0.02 mmol, 1.3 mg). The solution was kept at room temperature and the reaction progress was monitored by HPLC (method A) for 5 days (Figure S17, Table 1 entry 21). An HPLC peak (retention time 14.4 min) was observed in addition to the starting material (retention time 9.4 min). The peak at 14.4 min was identified by HPLC-ESI<sup>+</sup>MS (positive mode *m/z* 310.14 and 332.12 for [M+H<sup>+</sup>] and [M+Na<sup>+</sup>], respectively) and after separating non protonable products from a reaction carried out on a larger scale as the *N*-carbamoyl-dipeptide. To this aim, a 1 mM solution of dipeptide 1-LL was prepared by dissolving the free dipeptide (0.1 mmol, 26.6 mg) in 100 mM MES buffer (pH 6.5, 100 mL). Then the solution was added to a mixture of solid CuSO<sub>4</sub> (0.5 mmol, 79.80 mg) and KCN (1 mmol, 65.12 mg). After xx days.hours, the *N*-carbamoyl-dipeptide was separated from the starting material by ion exchange chromatography using Dowex 50 (H<sup>+</sup>) ion exchange resin. The resin was extensively eluted with pure water and the resulting aqueous solution was collected and freeze-dried giving a white solid residue (10 mg). <sup>1</sup>H NMR (300 MHz, D<sub>2</sub>O) δ<sub>H</sub> / ppm: 7.14 (d, *J* = 8.7 Hz, 2 H), 6.88 (d, *J* = 8.7 Hz, 2 H), 4.30 (dd, *J* = 8.2, 6.0 Hz, 1 H), 4.20 (q, *J* = 7.3 Hz, 1 H), 3.74 (s, 3 H), 3.02 – 2.91 (m, 1 H), 2.83 (dd, *J* = 14.0, 8.2 Hz, 1 H), 1.28 (d, *J* = 7.3 Hz, 3 H); HPLC analysis (method A): retention time 14.4 min (Figure S17, Table 1 entry 21).



**Figure S17** Reaction of 1 mM H-Tyr(Me)-L-Ala-OH **1-LL** with 5 mM CuSO<sub>4</sub> and 10 mM KCN in MES buffer (pH 6.5). HPLC chromatogram (method A, UV detection 273 nm) of **A**: the reaction medium after 5 days at room temperature; **B**: starting material **1-LL**; **C**: authentic sample of the stereoisomer H-Tyr(Me)-D-Ala-OH **1-LD**; **D**: authentic sample of *N*-carbamoyl-Tyr(Me)-L-Ala-OH.

### Reaction of 1 mM H-Tyr(Me)-L-Ala-OH **1-LL** in the presence of 10 mM DAMN

A 1 mM solution of H-Tyr(Me)-L-Ala-OH **1-LL** was prepared by dissolving the free dipeptide (0.05 mmol, 13.3 mg) in 100 mM MES buffer (pH 6.5, 50 mL). A 6 mL sample of the stock solution was added to (0.06 mmol, 6.3 mg) DAMN. The solution was kept at room temperature and the reaction progress was monitored by HPLC (method A) for 7 days (Figure S18, Table 1 entry 22) indicating the formation of a product with a 14.4 retention time identical to that of the *N*-carbamoyl dipeptide and another major non-identified product (retention time 18.8 min). The HPLC-ESI-MS (positive mode) confirmed the presence of the carbamoyl peptide  $m/z$  310.14 and 332.12 for [ $\text{M}+\text{H}$ ] $^+$  and [ $\text{M}+\text{Na}$ ] $^+$ , respectively and suggested the composition of an adduct of the dipeptide with cyanic acid and DAMN for the unknown derivative (calcd 418.1833 for C<sub>18</sub>H<sub>24</sub>N<sub>7</sub>O<sub>5</sub> $^+$  [ $\text{M}+\text{H}$ ] $^+$ , found 418.1800) (Figure S18, Table 1 entry 22).



**Figure S18** Reaction of 1 mM H-Tyr(Me)-L-Ala-OH **1-LL** with 10 mM diaminomaleonitrile (DAMN) in MES buffer (pH 6.5). HPLC chromatograms (method A, UV detection 273 nm) of **A**: the initial mixture and **B**: the reaction medium of 1 mM H-Tyr(Me)-L-Ala-OH **1-LL** with 10 mM DAMN in MES buffer at pH 6.5 after 7 days. H<sub>2</sub>NCO-Tyr(Me)-L-Ala-OH is an abbreviation of *N*-carbamoyl-Tyr(Me)-L-Ala-OH dipeptide.

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