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Electronic Supporting Information

Diastereoselectivity in prebiotically relevant 5(4*H*)-oxazolone-mediated peptide couplings

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1. General Experimental Conditions and Equipment

All reagents and solvents were purchased from commercial sources (Acros, Aldrich, Bachem) and were used without further purification. NMR spectra in DMSO-d_6 solution were recorded on a Bruker Avance 300 spectrometer at 300 MHz and 75.48 MHz for ^{1}H and ^{13}C, respectively. Chemical shifts were referred to Me_4Si as an internal reference. Data are presented as follows; chemical shift (δ_H or δ_C in ppm), multiplicity (s = singulet, d = doublet, t = triplet, q = quadruplet, dd = doublet of doublet, dt = doublet of triplet, m = multiplet, br = broad), coupling constant *J* (Hz) and integration. High-resolution mass spectra (HRMS) were recorded using a Waters Q-tof mass spectrometer using negative ion (ESI^-) or positive ion (ESI^+) electrospray ionisation mode. HPLC analyses were carried out on a Waters Alliance system including a E2695 separation module and a 2998 photodiode array detector; Column BDS Hypersil C18 (5μm, 2.1×150mm). Two elution methods were used: Method 1: flow 0.2 mL/min, solvent A 0.1% TFA in H_2O, solvent B 0.1% TFA in CH_3CN, gradient 5% B to 25% over 25 min; detection 273 nm; Method 2: as Method 1 except gradient 10% B to 30% over 25 min. Chiral HPLC analyses were carried out on a Beckman Coulter Gold HPLC System comprising a Solvent Delivery module 126 and a UV/VIS Detector 168 and using a Column CHIRALCEL® OJ-RH (5μm, 4.6×150mm); flow 0.8 mL/min, isocratic method with 1% TFA in H_2O / CH_3CN; (90:10); detection at 273 nm.

2. Synthesis

Dipeptides Ac-L-Tyr(Me)-Xaa-OH, 1.

Method A. In a first step, Boc-L-Tyr(Me)-OH was activated under the form of an ester of *N*-hydroxysuccinimide (HOSu). To this aim, Boc-L-Tyr(Me)-OH (1 g, 3.39 mmol) and HOSu (0.41 g, 3.55 mmol) were dissolved in THF (4 mL), the mixture was stirred at 0°C and dicyclohexylcarbodiimide (0.768 g, 3.72 mmol) was added. After 2 h at 0°C under stirring, the mixture was allowed to stand for 16 h at 4°C and the solid dicyclohexylurea was filtered off, washed with cold ethyl acetate and the filtrate was recovered. The solution was concentrated under vacuum and the solid residue was crystallized from AcOEt/hexane. The product was obtained almost quantitatively as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6), δ ppm: 7.70 (d, 1H, J^3 8.3 Hz) 7.35 (d, 2H, J^3 8.3 Hz), 6.9 (d, 2H, J^3 8.3 Hz), 4.57 (m, 1H), 3.80 (s, 3H), 2.9–3.3 (m, 6H), 1.44 (s, 9H).

The solid (Boc-L-Tyr(Me)-OSu) was subsequently reacted in CH_2Cl_2 (10 mL) with D- or L-alanine benzyl ester (0.75 g, 4.19 mmol) in the presence of DIEA (0.9 mL, 5.2 mmol). The mixture was stirred overnight at r.t. and concentrated. The residue was dissolved in ethyl acetate and the organic layer was washed with concentrated

Na_2CO_3, 1M $KHSO_4$, and brine, concentrated under reduced pressure. The Boc-protecting group was removed from the solid residue by treatment with TFA/H_2O (90:10) (10mL) for 20 min. The mixture was concentrated under reduced pressure, dissolved in water, brought to pH 10 with Na_2CO_3 and rapidly extracted with ethyl acetate. The organic layer was washed with brine, dried over Na_2SO_4 and concentrated under reduced pressure. The residue was dissolved CH_2Cl_2 (10 mL). Acetic anhydride (1.5 eq) was added to the solution with DIEA (1 eq) and the solution was stirred for 1 h at 0°C and 5 h at r.t. Ethyl acetate was added to the solution and the organic layer was washed with concentrated Na_2CO_3, 1M $KHSO_4$, and brine, concentrated under reduced pressure. The solid residue was recrystallized from ethyl acetate/hexane. The solid was dissolved in *i*PrOH/H_2O 70:30 (10 mL) and 1N NaOH (1.5 eq) was added to the solution 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 1M HCl and extracted with ethyl acetate. The organic layer was washed with brine, dried over Na_2SO_4 and concentrated to give the product as a solid residue.

Method B. A mixture of Boc-L-Tyr(Me)-OH (3.4 mmol) and HOBt (1.2 eq) was stirred in CH_2Cl_2 (10mL) at 0°C. DCC (1.0 eq) was added and the mixture was stirred at 0°C for 15 min and the aminoacid benzyl ester tosylate (1.2 eq) was added with DIEA (1.3 eq). The mixture was stirred at 0°C for 10 min and then allowed to react overnight at r.t. The DCU residue was filtered off, washed with CH_2Cl_2 and the filtrate was concentrated and dissolved in ethyl acetate (50 mL) and the organic layer was washed with concentrated Na_2CO_3, 1M $KHSO_4$, and brine, concentrated under reduced pressure. The Boc-protected dipeptide ester was then treated in a similar way as in method A.

Ac-L-Tyr(Me)-L-Ala-OH, L,L-1a. Method A. Yield: 80 mg, 15%; ^{1}H NMR (300 MHz, DMSO-d_6), δ ppm: 8.35 (d, 1H, J^3 8.3 Hz) 8.05 (d, 1H, J^3 8.3 Hz) 7.25 (d, 2H, J^3 8.3 Hz) 6.85 (d, 2H, J^3 8.3 Hz) 4.50 (m, 1H) 4.25 (m, 1H) 3.75 (s, 3H) 2.50-3.00 (m, 2H) 1.75 (s, 3H) 1.30 (d, 3H); ^{13}C NMR (DMSO-d_6), δ ppm: 175.6, 173.0, 170.6, 159.3, 131.7, 131.5, 115.0, 56.5, 55.5, 49.1, 38.4, 24.1, 18.8; HRMS (ESI) *m/z*: 309.1450 ($M+H^+$); HPLC (Method 1) retention time 14.8 min.

Ac-L-Tyr(Me)-D-Ala-OH, L,D-1a. Method B. Yield: 640 mg, 59%; ^{1}H NMR (300 MHz, DMSO-d_6), δ ppm: 8.25 (d, 1H, J^3 8.3 Hz) 8.00 (d, 1H, J^3 8.3 Hz) 7.10 (d, 2H, J^3 8.3 Hz) 6.80 (d, 2H, J^3 8.3 Hz) 4.50 (m, 1H) 4.20 (m, 1H) 3.70 (s, 3H) 2.50-3.00 (m, 2H) 1.75 (s, 3H) 1.20 (d, 3H). ^{13}C NMR (DMSO-d_6), δ ppm: 174.4, 171.4, 169.3, 158.2, 130.6, 130.2, 113.8, 55.4, 54.3, 47.8, 33.8, 22.9, 17.8; HRMS (ESI) *m/z*: 309.1450 ($M+H^+$); HPLC (Method 1) retention time 17.6 min.

Ac-L-Tyr(Me)-L-Val-OH, L,L-1b. Method B. Yield: 720 mg, 54%; ^{1}H NMR (300 MHz, DMSO-d_6), δ ppm: 7.85 (d, 2H, J^3 8.3 Hz) 7.00 (d, 2H, J^3 8.3 Hz) 6.55 (d, 2H, J^3 8.3 Hz) 4.45 (m, 1H) 3.95 (m, 1H) 3.50 (s, 3H) 2.40-2.80 (m, 2H) 1.55 (s, 3H) 0.75 (m, 6H). ^{13}C NMR (DMSO-d_6), δ ppm: 173.3, 172.3, 169.6, 158.2, 130.6, 130.3, 113.8, 60.2, 57.6, 55.3, 54.3, 37.1, 31.1, 30.3, 22.8, 19.5, 18.4, 14.5; HRMS (ESI) *m/z*: 337.1763 (M+H$^+$); HPLC (Method 1) retention time 20.7 min.

Ac-L-Tyr(Me)- L-Leu-OH, L, L-1c. Method B. Yield: 970 mg, 66%; ^{1}H NMR (300 MHz, DMSO-d_6), δ ppm: 8.25 (d, 1H, J^3 8.3 Hz) 8.00 (d, 1H, J^3 8.3 Hz) 7.20 (d, 2H, J^3 8.3 Hz) 6.80 (d, 2H, J^3, 8.3 Hz) 4.55 (m, 1H) 4.25 (m, 1H) 3.70 (s, 3H) 2.50-3.00 (m, 2H) 1.80 (s, 3H) 1.40-1.80 (m, 3H) 0.90 (m, 6H); ^{13}C NMR (DMSO-d_6), δ ppm: 174.4, 172.1, 169.5, 158.2, 130.6, 130.3, 113.8, 60.2, 55.3, 54.3, 50.68, 37.2, 31.1, 24.7, 23.3, 22.9, 21.8, 21.2, 14.5; HRMS (ESI) *m/z*: 351.1920 (M+H$^+$); HPLC (Method 2) retention time 20.3 min.

Ac-L-Tyr(Me)-OH, 3. A mixture of H-Tyr(Me)-OH (0.976 g, 5 mmol) and Na_2CO_3 was suspended in dioxane (5 mL) and water (10 mL) and maintained under vigorous stirring at 0°C. Then 1 mL of a 5 mL solution of acetic anhydride (0.52 mL, 5.5 mmol) in dioxane and 2 mL of a 10 mL solution of NaOH (6.5 mmol) in water were added. The rest of the solution were added in four equivalent portions every 10 min. The reaction was allowed to reach completion at r.t. for 2 h. The solution was acidified to pH 2 with 1N HCl and extracted with ethyl acetate (2 × 50 mL). The organic layer was recovered, washed with brine, dried over Na_2SO_4 and concentrated under reduced pressure. The oily residue was allowed to crystallize for 16 h at 4°C in the minimum volume of ethyl acetate and hexane. A white solid was collected, washed with hexane and dried. Yield: 813 mg, 69%; ^{1}H NMR (300 MHz, DMSO-d_6), δ ppm: 12.60 (s, 1H); 8.15 (d, 1H); 7.15 (d, 2H); 6.80 (d, 2H); 4.35 (m, 1H); 3.70 (s, 3H); 2.70–3.00 (m, 2H); 1.80 (s, 3H); ^{13}C NMR (DMSO-d_6), δ ppm: 174.28, 170.23, 158.93, 131.11, 130.58, 56.01, 54.81, 23.41; HRMS *m/z* 238.1073 (M+H$^+$).

(4-Methoxybenzyl)-2-methyl-5(4*H*)-oxazolone, 4. Ac-L-Tyr(Me)-OH **3** (500 mg, 2.11 mmol) was dissolved in CH_2Cl_2 (20 mL) and stirred at 0°C and EDC (445 mg, 2.32 mmol) was added. The mixture was stirred for 1h at 0°C. CH_2Cl_2 (20 mL) was added and the organic layer was washed with water, saturated $NaHCO_3$, saturated NaCl, dried over Na_2SO_4. The solution was concentrated under reduced pressure and the solid residue (>95%) was stored at –18°C and used without further purification. ^{1}H NMR (300 MHz, DMSO-d_6), δ ppm: 7.15 (d, 2H); 6.80 (d, 2H); 4.60 (m, 1H); 3.70 (s, 3H); 2.70–3.10 (m, 2H); 2.10 (s, 3H); ^{13}C NMR (DMSO-d_6), δ ppm: 178.84, 169.65, 162.10, 130.84, 128.33, 65.95, 55.41, 35.64, 15.05.

This synthesis protocol was shown to lead to the racemization of the product: 100 μL of a 100 mM solution of **4** in acetonitrile were diluted to 10 mL with pure water and the solution allowed to stand at r.t. for 12 h. The resulting Ac-Tyr(Me)-OH solution was analysed by chiral HPLC indicating the presence of a 1:1 mixture of L- and D-enantiomers with retention times of 8.0 and 9.3 min, respectively.

Ac-L-Tyr(Me)-L-Ala-NH_2. H-L-Ala-NH_2·HCl (0.137 g, 1.1 mmol) was dissolved in *i*Pr-H_2O (7:3, 13 mL), $NaHCO_3$ (0.10 g, 1.2 mmol) was added. The mixture was stirred at r.t. and Boc-L-Tyr(Me)-OSu (0.39 g, 1 mmol), prepared from Boc-L-Tyr(Me)-OH (see above method A, p. S2) was added to the solution. The mixture was stirred 48 h and then concentrated under reduced pressure, redissolved in water and twice extracted with ethyl acetate (50 mL). The organic layers were washed with concentrated Na_2CO_3, 1M $KHSO_4$, and brine, collected and concentrated under reduced pressure. The Boc-protecting group was removed with TFA (2 mL). Cold ethyl ether (50 mL) was added and the mixture was extracted with water (30 mL). The aqueous layer was collected and washed with ethyl ether (4 × 50 mL). The aqueous layer was concentrated under reduced pressure and then freeze-dried to yield a white solid. The residue was reacted with acetic anhydride (1.5 eq) and DIEA (4 eq) in CH_2Cl_2 (5 mL) at 0°C for 1 h and 5 h at r.t. The solvent was removed under reduced pressure and the residue was dissolved in water and introduced on a Dowex-50 ion exchange resin column (H+-form), the column was eluted with water and the resulting aqueous solution was freeze-dried. The residue was purified by liquid chromatography on silicagel (eluent CH_2Cl_2/MeOH, 95:5 and then 90:10) to give a solid. Yield: 80 mg, 26%; ^{1}H NMR (300 MHz, DMSO-d_6), δ ppm: 7.95 (m, 2H) 7.10 (m, 3H) 6.95 (s, 1H) 6.80 (m, 3H) 4.00-4.50 (m, 2H) 3.60 (s, 3H) 2.50-3.00 (m, 2H) 1.75 (s, 3H) 1.15 (s, 3H); ^{13}C NMR (DMSO-d_6), δ ppm: 173.9, 170.9, 169.2, 157.7, 130.1, 129.8, 113.4, 54.9, 54.2, 47.9, 36.6, 22.4, 18.3 HRMS *m/z* : 308.1613.

Ac-L-Tyr(Me)-D-Ala-NH_2. The Ac-L-Tyr(Me)-D-Ala-NH_2 isomer was prepared in a similar way from H-D-Ala-NH_2·HCl (70 mg, 0.56 mmol) and Boc-L-Tyr(Me)-OSu (200 mg, 0.50 mmol) using a similar procedure. Yield: 48 mg, 31% ^{1}H NMR (300 MHz, DMSO-d_6), δ ppm: 8.05 (m, 2H) 7.05 (m, 3H) 6.95 (s, 1H) 6.75 (m, 3H) 4.00-4.50 (m, 2H) 3.65 (s, 3H) 2.50-3.00 (m, 2H) 1.70 (s, 3H) 1.05 (s, 3H) HRMS *m/z* : 308.1610.

Ac-L-Tyr(Me)-L-Ala-Gly-NH_2. The peptide was prepared by standard solid-phase peptide synthesis procedures from aminomethylpolystyrene resin (0.56 mmol/g -NH_2, 0.44 g) by Fmoc-strategy using a Rink-amide linker. Yield: 35 mg, 39% ^{1}H NMR (300 MHz, DMSO-d_6), δ ppm: 8.30 (d, 1H) 8.10 (d, 1H) 7.95 (d, 1H) 7.10-7.20 (m, 4H) 6.80 (d, 2H) 4.00-4.50 (m, 2H) 3.60 (m, 5H) 2.50-3.00 (m, 2H) 1.75 (s, 3H) 1.15 (s, 3H)

^{13}C NMR (DMSO-d_6), δ ppm: 172.7, 172.1, 171.3, 169.8, 158.2, 130.6, 130.3, 113.9, 55.4, 54.7, 48.9, 42.3, 39.1, 22.9, 18.2.

3. EDC-promoted epimerization of dipeptides Ac-L-Tyr(Me)-Xaa-OH, 1a-c.

The dipeptides **1a**-**c** (0.01 mmol) were dissolved in a 0.1 M MES buffer (10 mL, pH 5.5 or pH 6.5). The solution was stirred at r.t. and solid EDC was added to the solution in 1 eq portions (0.01 mmol) every 24 hours. The addition was repeated four times until no change in the diastereomeric ratio could be observed at pH 5.5. The steady state was not reached at pH 6.5 owing to slower rates. The extent of epimerization was monitored by HPLC (detection) of the two diastereomers as the only products identified by HPLC (Figure S1, Table S1).

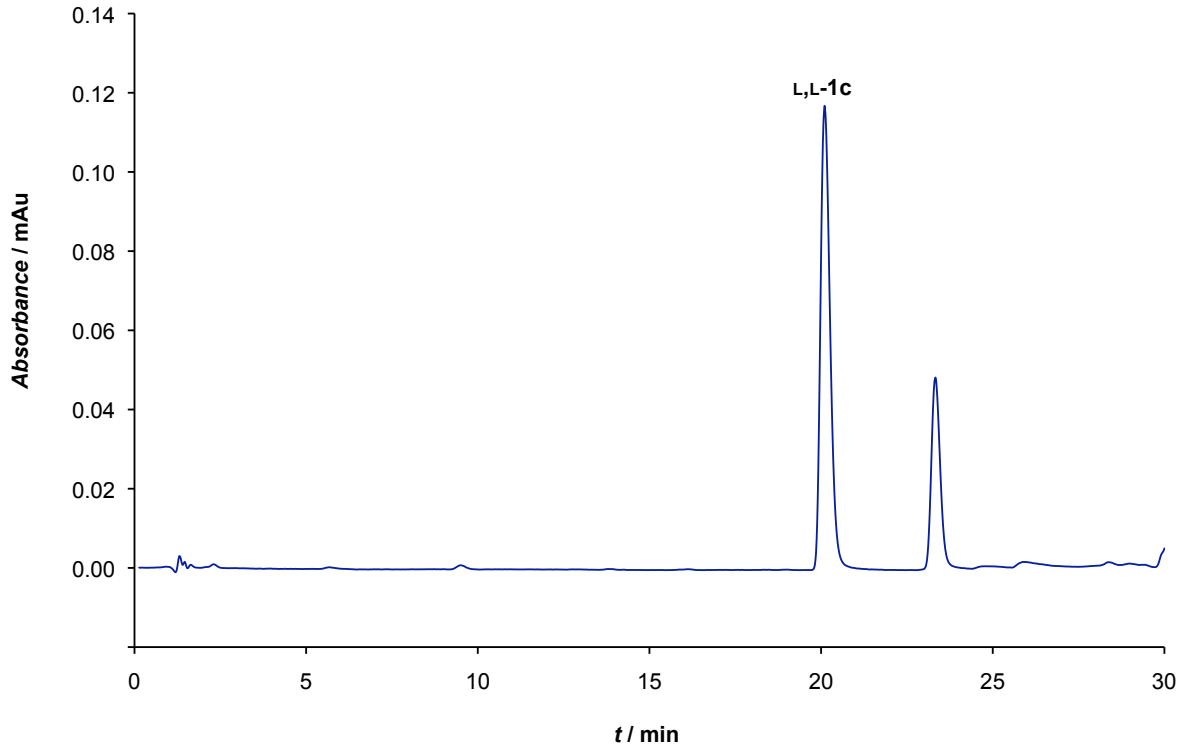


Figure S1: EDC-promoted epimerization of Ac-L-Tyr(Me)-L-Leu-OH, **L,L-1c**. HPLC analysis (Method 1) of the diastereomeric mixture in the reaction medium after reaction completion (4 days).

Table S1. HPLC behaviour of diastereomers.

Compound number	Sequence	HPLC method	Retention time / min	Identification method
L,L-1a	Ac-L-Tyr(Me)-L-Ala-OH	1	14.8	Authentic sample
L,D-1a	Ac-L-Tyr(Me)-D-Ala-OH	1	17.6	Authentic sample
L,L-1b	Ac-L-Tyr(Me)-L-Val-OH	1	20.7	Authentic sample
L,D-1b	Ac-L-Tyr(Me)-D-Val-OH	1	25.9	HPLC-MS (*m/z* 337.2)
L,L-1c	Ac-L-Tyr(Me)-L-Leu-OH	2	20.3	Authentic sample
L,D-1c	Ac-L-Tyr(Me)-D-Leu-OH	2	23.5	Fig. S1

4. Coupling Ac-L-Tyr(Me)-L-Ala-OH and Ac-L-Tyr(Me)-D-Ala-OH with H-Gly-NH_2.

EDC (10 μM, 1 eq) was added to a 1mM of **L,L-1a** or **L,D-1a** and 10mM glycinamide solution in pH 5.5 or pH 6.5 MES buffer (10 mL). The extent of epimerization was monitored by HPLC (Method 1). The resulting yields and diastereomeric ratio are displayed in Table S2. A 60/40 mixture of the diastereomers (Entry 3) was additionally used as a reactant to confirm the steady state value of *d.r.*

Table S2. Epimerization of the C-terminal residue of dipeptides Ac-L-Tyr(Me)-D/L-Ala-OH (1mM in 0.1M MES buffer pH 5.5 and 6.5 at r.t.) upon coupling with 10 mM H-Gly-NH_2 promoted by 1 mM EDC. Values of the diastereoisomeric ratio of the product (homochiral/heterochiral).

Entry	Reactant	Product diastereomeric ratio[a] (yield[b])	
		pH 5.5	pH 6.5
1	Ac-L-Tyr(Me)-D-Ala-OH	47 : 53 (40%)	44 : 56 (35%)
2	Ac-L-Tyr(Me)-L-Ala-OH	55 : 45 (58%)	60 : 40 (28%)
3	Ac-L-Tyr(Me)-L-Ala-OH (60%) + Ac-L-Tyr(Me)-D-Ala-OH (40%)	57 : 43 (55%)	58 : 42 (35%)

[a] Determination by integration of HPLC peaks (method 1, detection at 273 nm) of Ac-L-Tyr(Me)-L-Ala-Gly-NH_2 (10.7 min) and Ac-L-Tyr(Me)-D-Ala-Gly-NH_2 (12.8 min); [b] Determination by comparison with peaks of Ac-L-Tyr(Me)-L-Ala-OH (13.7 min), Ac-L-Tyr(Me)-D-Ala-OH (16.8 min),.

5. EDC-promoted coupling of Ac-L-Tyr(Me)-OH with nucleophiles (Coupling method A)

General procedure. Ac-L-Tyr(Me)-OH (0.01 mmol) and the nucleophilic reactant (0.1 mmol) were dissolved in 0.1M MES (pH 5.5, pH 6.5, 6.8) or HEPES (pH 7.5) buffers (10 mL). The solution was stirred at r.t. and solid EDC (0.01 mmol, 1 eq) was added to the solution. 1-mL aliquots were withdrawn and the extent of reaction was monitored by HPLC (Method 1 except for H-Leu-OH for which Method 2 was used) by comparison with Ac-L-Tyr(Me)-OH (15.1 min, and 9.2 min using Method 2).

Assessment of the reaction rates for coupling racemic alaninamide. The overall extent of reaction was determined from the areas of HPLC peaks of homochiral and heterochiral diastereomer of Ac-Tyr(Me)-Ala-NH_2 (11.2 and 13.4 min, respectively, Method 1) and starting material Ac-L-Tyr(Me)-OH (15.1 min). The reaction progress is displayed in Figure S2. HPLC MS: *m/z* 308.1 (homochiral dipeptide), 308.1 (heterochiral dipeptide), 238.1 (starting material).

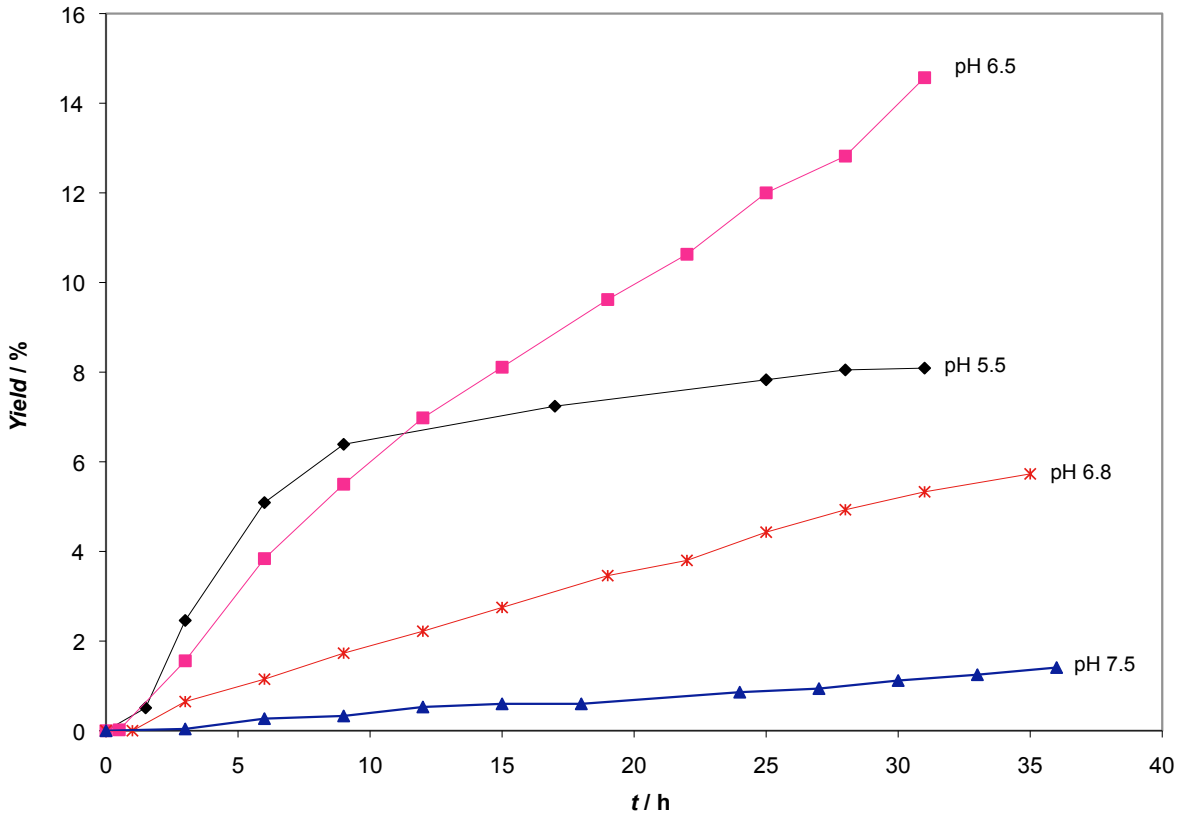


Figure S2: The reaction of 1mM Ac-Tyr(Me)-OH with 10mM D/L-alaninamide promoted by 1mM EDC in 0.1M MES (pH 5.5, 6.5, 6.8) or HEPES (pH 7.5) buffers. Conversion yield (HPLC determination, Method 1) referring to the sum of the yields of diastereomers.

Influence of pH on the reaction of Ac-L-Tyr(Me)-OH 3 with H-L-Ala-NH_2 and H-D-Ala-NH_2. The enantiomers of H-Ala-NH_2·HCl in 10 mM concentration were allowed to react with a 1mM solution of Ac-L-Tyr(Me)-OH **3** in 0.1M MES (pH 5.5, pH 6.5, 6.8) or HEPES (pH 7.5) buffer (10 mL, MES at pH 5.5, pH 6.5, 6.8 and HEPES at pH 7.5) according to method A. The overall extent of reaction and *d.r.* (Table S3) were calculated from the areas of HPLC peaks of homochiral and heterochiral diastereomer (11.2 and 13.4 min, respectively) and starting material Ac-L-Tyr(Me)-OH (15.1 min). The results are displayed in Table S3.

Table S3: Influence of the pH on the *d.r.* (ratio of the HPLC areas of homochiral vs. heterochiral products) and conversion yield (determined from the HPLC area of both dipeptides) in the reaction of Ac-Tyr(Me)-OH with L- or D-alanineamide with EDC.

pH	Product diastereomeric ratio (conversion yield)	
Starting material	L-Ala-NH_2	D-Ala-NH_2
5.5	66 / 34 (8%)	43 / 57 (6%)
6.1	67 / 33 (13%)	65 / 35 (13%)
6.5	72 / 28 (15%)	73 / 27 (12%)
6.8	75 / 25 (10%)	76 / 24 (10%)
7.5	77 / 23 (2%)	78 / 22 (2%)

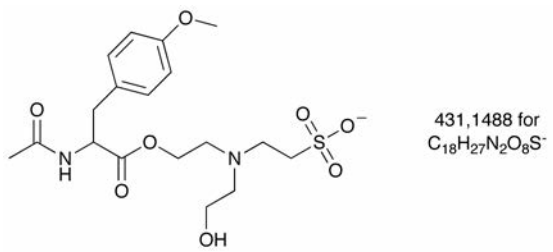
Reaction with other nucleophiles.

Reactions were carried out in a similar way as above from 1mM solution of Ac-L-Tyr(Me)-OH and 10 mM nucleophile concentrations. The results are displayed in Table 2 (main Article) and Table S4.

6. Coupling 5(4*H*)-oxazolone 4 with nucleophiles (Coupling method B)

General procedure. Oxazolone **4** (0.01 mmol) was dissolved in a solution of the nucleophilic reactant (10 mM) in 0.1M MES (pH 5.5, pH 6.5, 6.8) or MOPS (pH 7.5) buffers (10 mL). 1 mL aliquots were withdrawn and the extent of reaction was monitored by HPLC (method 1 and 2 for H-Leu-OH) by comparison with Ac-L-Tyr(Me)-OH (15.104 min).

In the experiments carried out at pH 7.5, MOPS was used instead of HEPES that proved to undergo a reaction with oxazolone **4** giving an adduct identified by HPLC-MS (retention time 15.0 min; *m/z* 529.13; ESI negative mode) and corresponding very probably to an ester with the available hydroxyl group of HEPES. No reaction of this kind was observed in MOPS buffer.



Influence of buffer concentration. The reaction of oxazolone **4** (1 mM) with L-or D-alaninamide (10 mM) was carried out at three different buffer concentrations, namely,

100 mM, 50 mM and 25 mM MES pH 6.5, and in the absence of buffer (pH maintained by the buffering property of H-Ala-NH_2). No effect of the buffer concentration on the *d.r.* was observed.

Table S4: Influence of the pH on the *d.r.* (ratio of the HPLC areas of homochiral vs. heterochiral products) and conversion yield (determined from the HPLC area of both products) in the reactions of Ac-Tyr(Me)-OH promoted by EDC (Coupling method A) or of 5(4H)-oxazolone (Coupling method B) with nucleophiles. (Continuation of Table 2 in main Article)

Entry	Nucleophile	Conditions (pH, t)	Activation method	*d.r.*	Conversion yield
21	H-L-Ala-NH2	pH 5.5, r.t.	B	56 / 44	9%
22	H-D-Ala-NH2	pH 5.5, r.t.	B	56 / 44	9%
23	H-D-Ala-NH2	pH 5.5, 0 °C	B	60 / 40	8%
24	H-D-Ala-OH	pH 5.5, r.t.	B	54 / 46	10%
25	H-L-Ala-OH	pH 5.5, r.t.	A	57 / 43	7%
26	H-D/L-Ala-OH	pH 5.5, r.t.	A	55 / 45	10%
27	H-L-Val-OH	pH 5.5, r.t.	A	53 / 47	7%
28	H-D-Val-OH	pH 5.5, r.t.	A	50 / 50	7%
29	H-L-Val-OH	pH 5.5, r.t.	B	49 / 51	9%
30	H-L-Leu-OH	pH 5.5, r.t.	A	57 / 43	7%
31	H-D-Leu-OH	pH 5.5, r.t.	A	54 / 46	7%
32	H-L-Leu-OH	pH 5.5, r.t.	B	47 / 53	8%
33	H-L-Ala-L-Ala-OH	pH 5.5, r.t.	B	58 / 42	9%
34	H-L-Ala-OH	pH 6.5, r.t.	A	72 / 28	12%
35	H-L-Ala-L-Ala-OH	pH 6.5, r.t.	B	69 / 31	26%
36	H-L-Val-OH	pH 6.5, r.t.	A	63 / 37	6%
37	H-D-Val-OH	pH 6.5, r.t.	A	61 / 39	6%
38	H-L-Val-OH	pH 6.5, r.t.	B	61 / 39	13%
39	H-L-Leu-OH	pH 6.5, r.t.	A	62 / 38	5%
40	H-D-Leu-OH	pH 6.5, r.t.	A	60 / 40	5%
41	H-L-Leu-OH	pH 6.5, r.t.	B	56 / 44	16%
42	H-D-Ala-OH	pH 7.5, r.t.	B	71 / 29	47%
43	H-L-Val-OH	pH 7.5,r.t.	B	66 / 34	27%
44	H-L-Leu-OH	pH 7.5, r.t.	B	59 / 41	32%

Identification of the diastereomers formed from alanine oligomers. The isomers obtained by addition of H–(L-Ala)$_n$–OH (n = 2–4) oligopeptides (Main Article, Table 2, entries 15–17, 19–20) were identified HPLC by comparison with the retention times of mixtures resulting from the reaction of Ac-Tyr(Me)–OSu (previously prepared under conditions limiting the epimerization) with the commercial L-alanine oligomers. To this aim, Ac-Tyr(Me)-OH **3** (50 mg, 0.21 mmol) and *N*-hydroxysuccinimide (HOSu,

25.5 mg, 1.05 eq) were introduced in THF (1 mL). The mixture was cooled to 0°C and DCC (47.7 mg, 1.1 eq) was added. The mixture was stirred for 2 h. The dicyclohexylurea precipitate was filtered off and washed with AcOEt. The filtrate was concentrated yielding a crude solid (90%). ^{1}H NMR (300 MHz, DMSO-d_6), δ ppm: 8.55 (d, 1H) 7.24 (d, 2H) 6.86 (d, 3H) 4.80 (m, 1H) 3.73 (s, 3H) 2.80-3.20 (m, 2H) 2.83 (s, 4H) 1.80 (s, 3H); The almost pure Ac-Tyr(Me)–OSu crude solid was used without further purification in subsequent coupling reactions. The reaction with Ala oligomers was performed from Ac-Tyr(Me)-OSu (0.33 mg, 1 μmol) and the H–(L-Ala)$_n$–OH oligomer (1.5 eq) in 100 μL of a 100 mM MES buffer in MeCN/water (1:1 v/v). After 30 min, the reaction was interrupted by dilution with water (900 μL) (longer reaction times were shown to decrease significantly the d.r.) and the solution was analysed by HPLC (Method 1).

Table S5: Determination of HPLC behaviour of Ac-D-Tyr(Me)-(Ala)$_n$-OH and Ac-D-Tyr(Me)-(Ala)$_n$-OH by the coupling of the hydroxysuccinimidyl ester of Ac-L-Tyr(Me) –OH.

Starting material	Ac-L-Tyr(Me)-(Ala)$_n$-OH Retention time / min	Ac-D-Tyr(Me)-(Ala)$_n$-OH Retention time / min	Diastereoisomeric ratio (Conversion yield)
H-Ala$_2$-OH	15.8	16.2	90 / 10 (77%)
H-Ala$_3$-OH	16.3	17.4	94 / 6 (87%)
H-Ala$_4$-OH	17.2	18.8	79 / 21 (34%)[a]

[a] Low yield resulting from the low solubility of H-Ala$_4$-OH in the medium.