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Supporting Information

Speeding up sustainable solution-phase peptide synthesis using T3P® as green coupling reagent: methods and challenges

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General methods

Unless otherwise stated, all materials, solvents and reagents were obtained from commercial suppliers and used without further purification. High-performance liquid chromatography (HPLC) reagent grade solvents were used. Specifically, Benzyloxycarbonyl (Z), tert-butyloxycarbonyl (Boc) amino acids, diisopropylethylenamine (DIPEA), pyridine (Py), triethylamine (TEA), 1,1,3,3-tetramethylguanidine (TMG), 2,6-lutidine, 1,5-diazabiciclo(5.4.0)undec-7-ene (DBU), tert-buthylamine ($^{t}BuNH_2$), morpholine, diethylaminopropylamine (DEAPA), Oxyma Pure® and *N,N'*-diisopropylcarbodiimide (DIC) were supplied by Iris Biotech, Merck or Fluorochem. Ethyl Acetate, *N*-buthyl pyrrolidone (NBP), dimethyl carbonate (DMC), *N*-octyl pyrrolidone (NOP), γ-valerolactone, acetonitrile (ACN), detrahydrofuran (THF), 1-methyl tetrahydrofuran, dicloromethane (DCM), isopropyl alcohol ($^{i}PrOH$), isopropyl acetate ($^{i}PrOAc$) and HPLC-quality ACN were purchased from Merck. Trifluoroacetic acid (TFA), triisopropyl silane (TIPS) and diisopropyl ether (DIPE) were supplied by Iris Biotech and Merck. The solvents, bases and coupling reagents were individually injected in HPLC using the same analytical methods employed for the evaluation of reactions progress, to establish their retention time. T3P® (50 wt. % in EtOAc) were supplied by Curia Global. ^{1}H-NMR and ^{13}C-NMR spectra were recorded with an INOVA 400 MHz instrument with a 5 mm probe. All chemical shifts were quoted relative to deuterated solvent signals. Distillations were performed with an Edwards RV3 vacuum pump. Z group removal was performed using a continuous-flow reactor (H-Cube® mini plus commercialized by ThalesNano Inc). $Pd/C_{(10\%)}$ filled cartridges (30 mm long) were supplied by ThalesNano Inc.

Analytical methods

HPLC-MS analyses were performed on Agilent 1260 Infinity II system coupled to ESI mass spectrometer (positive-ion mode, m/z = 100–3000 amu, fragmentor 30 V), with the following parameters:

- column Phenomenex Luna C18 5 µm, 250 x 4.6 mm
- temperature: 35°C
- injection volume: 10 µL
- UV: 220 nm or 210 nm
- mobile phases: H_2O+0.08%TFA (mobile phase A) and ACN+0.08%TFA (mobile phase B)
- flow: 0.5 mL/min or 1.0 mL/min

The gradient of analytical methods reported across the paper are defined as follow:

Method 1		
Flow: 1 ml/min		
Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	80	20
5	60	40
25	60	40
30	80	20

Method 2		
Flow: 1 ml/min		
Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	80	20
15	60	40
20	60	40
35	80	20

Method 3		
Flow: 1 ml/min		
Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	80	20
15	20	80
22	20	80
30	80	20

Method 4		
Flow: 0.5 ml/min		
Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	80	20
10	60	40
20	60	40
25	70	30
30	80	20

ChemStation software was used for data processing. Percentage areas of integrated peaks are reported in mAu.

1. Conditions screening for model dipeptide synthesis

General procedure

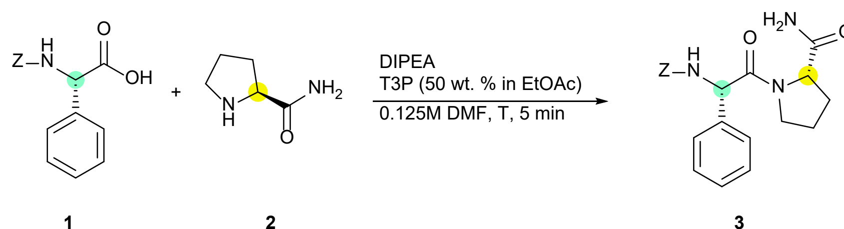


Figure S1. Optimization of conditions for the synthesis of model peptide (**3**).

In an oven-dried Schlenk purged under N_2 atmosphere, Z-Phg-OH (**1**) (29 mg, 0.1 mmol, 1.0 eq.) and H-Pro-NH_2 (**2**) (11 mg, 0.1 mmol, 1.0 eq.) were dissolved in DMF (0.125M). DIPEA and T3P® (50 wt.% in EtOAc) were added sequentially as reported in Table S1, stirring the reaction at 0 °C or room temperature for 5 minutes. The conversion was monitored by HPLC-MS analysis using Method 1 (see Analytical methods section reported above).

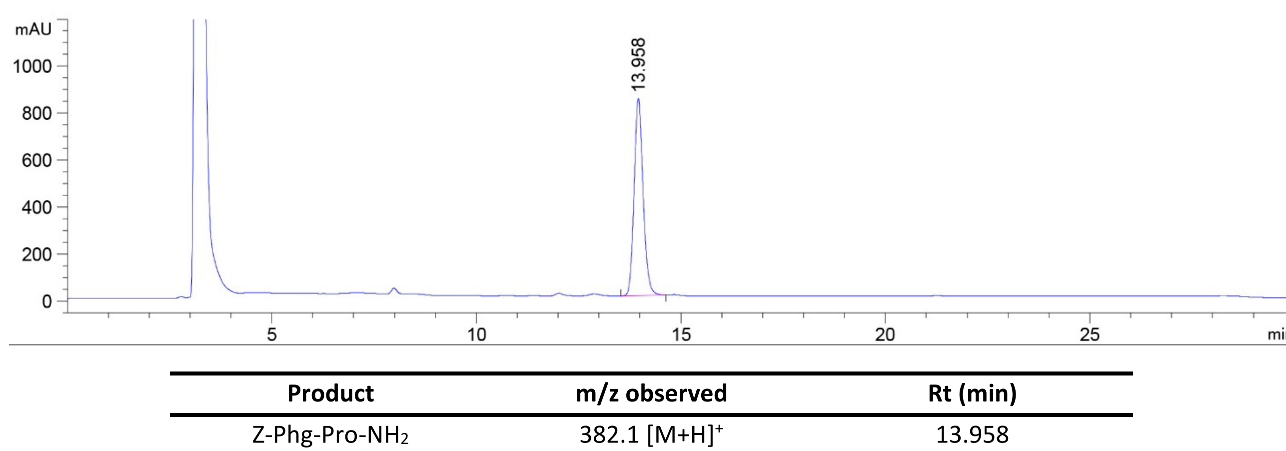
HPLC chromatograms

Peak at 3.2 min is related to DMF.

Table S1. Conditions screening.

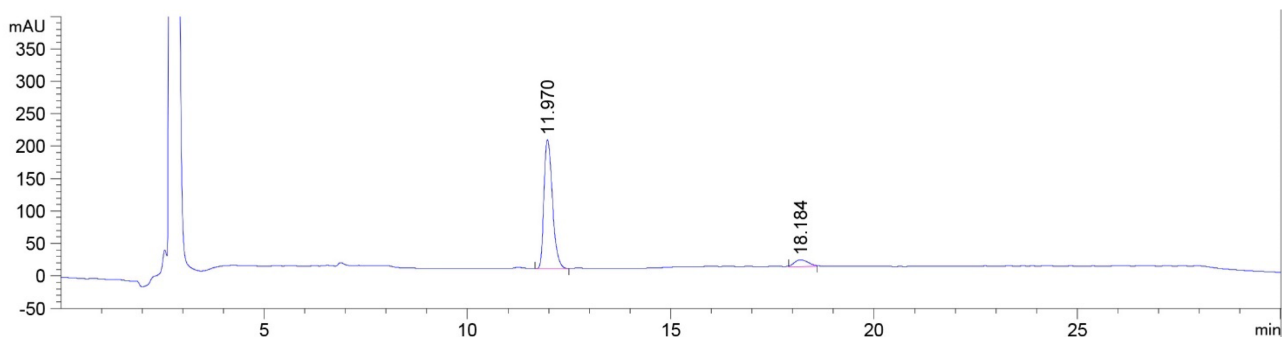
Entry[a]	T3P® (equiv)	Base (equiv)	Conversion (%)
1	1	DIPEA (1)	81
2	1	DIPEA (2)	88
3	1.5	DIPEA (3)	>99
4[b]	1.5	DIPEA (3)	93

[a]Reactions were performed by dissolving Z-Phg in DMF (0.1 M conc) under nitrogen atmosphere and adding reagents in the following order: Pro-NH_2, DIPEA and finally T3P®. [b]Reaction was performed at 0°C.



Product	m/z observed	Rt (min)
Z-Phg-Pro-NH_2	382.1 $[M+H]^+$	13.958

Figure S2. Chromatogram of Z-Phg-Pro-NH_2 (**3**) in DMF at 220 nm (entry 3, Table S1).



Product	m/z observed	Rt (min)	Area (%)
Z-Phg-Pro-NH_2	381.7 $[M+H]^+$	11.970	92.7719
Z-Phg-OH (**1**)	286.1 $[M+H]^+$	18.184	7.2281

Figure S3. Chromatogram of Z-Phg-Pro-NH_2 (**3**) in DMF at 220 nm (entry 4, Table S1).

2. Bases screening for coupling step

General procedure

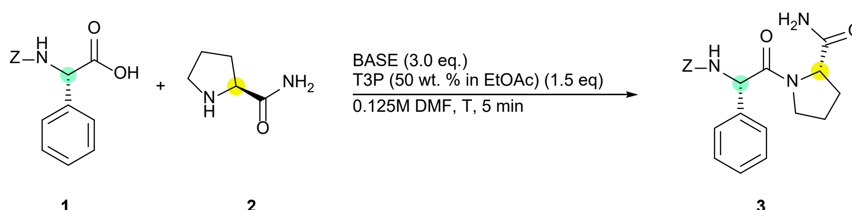


Figure S4. Synthesis of model peptide testing several bases.

In an oven-dried Schlenk purged under N_2 atmosphere, Z-Phg-OH (**1**) (29 mg, 0.1 mmol, 1.0 eq.) and H-Pro-NH_2 (**2**) (11 mg, 0.1 mmol, 1.0 eq.) were dissolved in DMF (0.125M). Subsequently, the desired base (0.3 mmol, 3.0 eq.) and T3P® (50 wt. % in EtOAc, 89 µL, 0.15 mmol, 1.5 eq.) were added at room temperature following this order as reported in Table S2. The solution was stirred at room temperature for 5 minutes and the conversion was monitored by HPLS-MS analysis (Analysis Method 1 in Analytical methods).

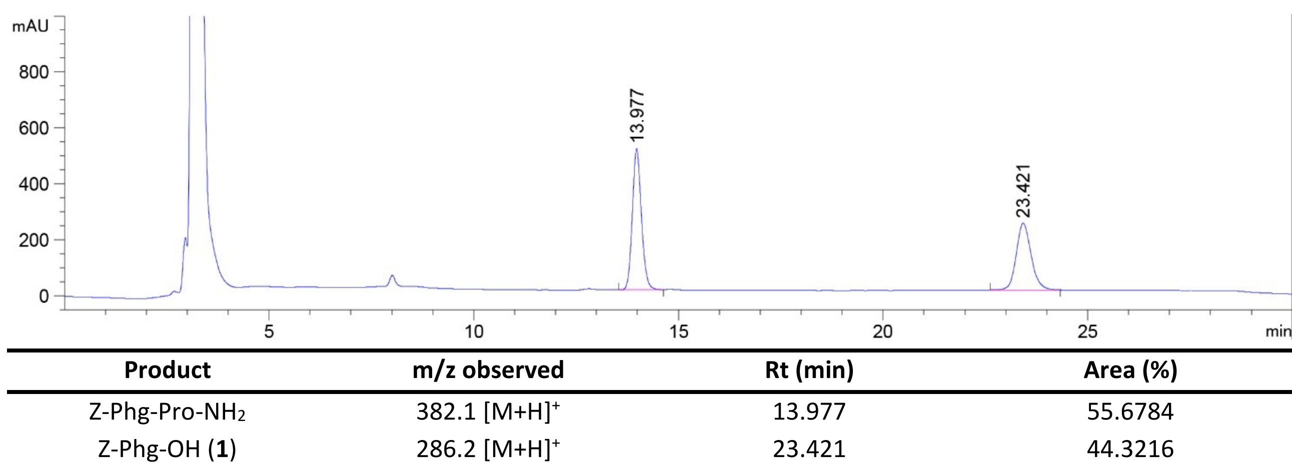
HPLC chromatograms

Peak at 3.2 min is related to DMF.

Table S2. Bases screening.

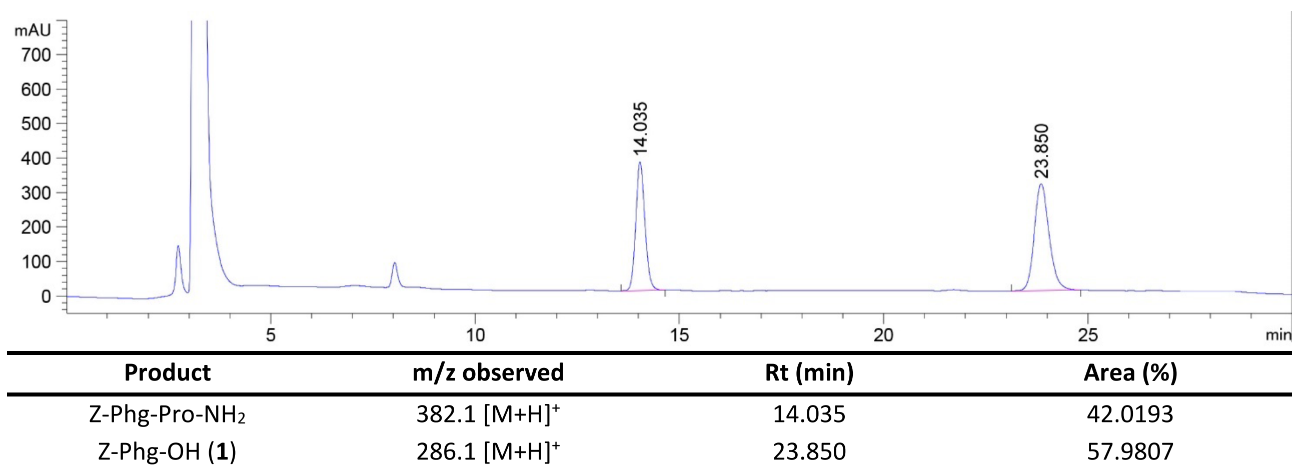
Entry[a]	T3P® (equiv)	Base		Conversion (%)
			greenness score	
1	1.5	2,6-lutidine	8.3	56
2	1.5	Pyridine	7.5	42
3	1.5	TEA	6.9	94
4	1.5	NMM	6.9	90
5	1.5	^{t}BuNH$_2$	6.5	93

[a]The reactions were performed under the conditions used in entry 3, Table S1, and conversion evaluated after 5 minutes.



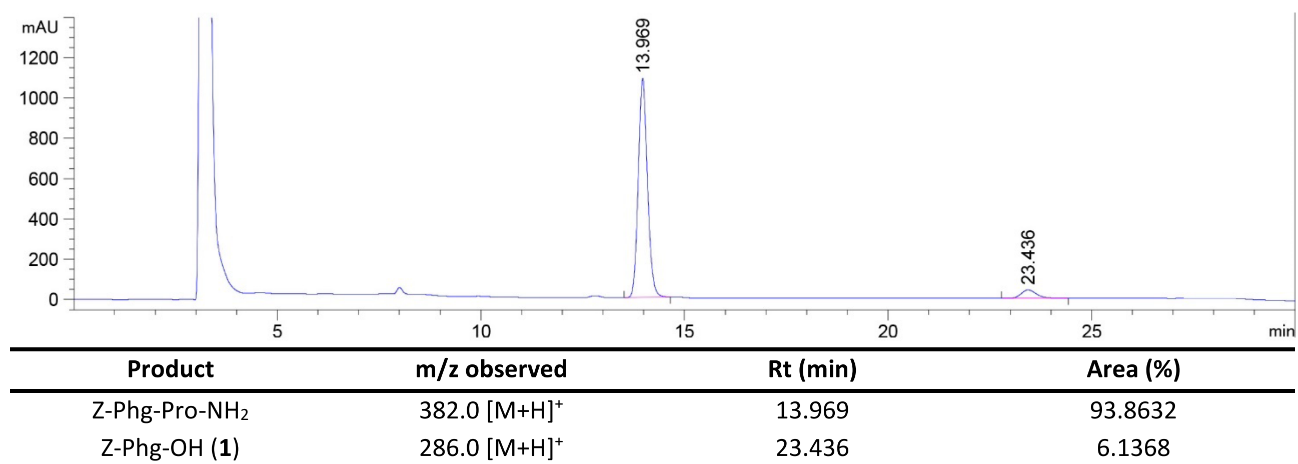
Product	m/z observed	Rt (min)	Area (%)
Z-Phg-Pro-NH$_2$	382.1 [M+H]$^+$	13.977	55.6784
Z-Phg-OH (**1**)	286.2 [M+H]$^+$	23.421	44.3216

Figure S5. Chromatogram of Z-Phg-Pro-NH$_2$ **(3)** at 220 nm (entry 1, Table S2).



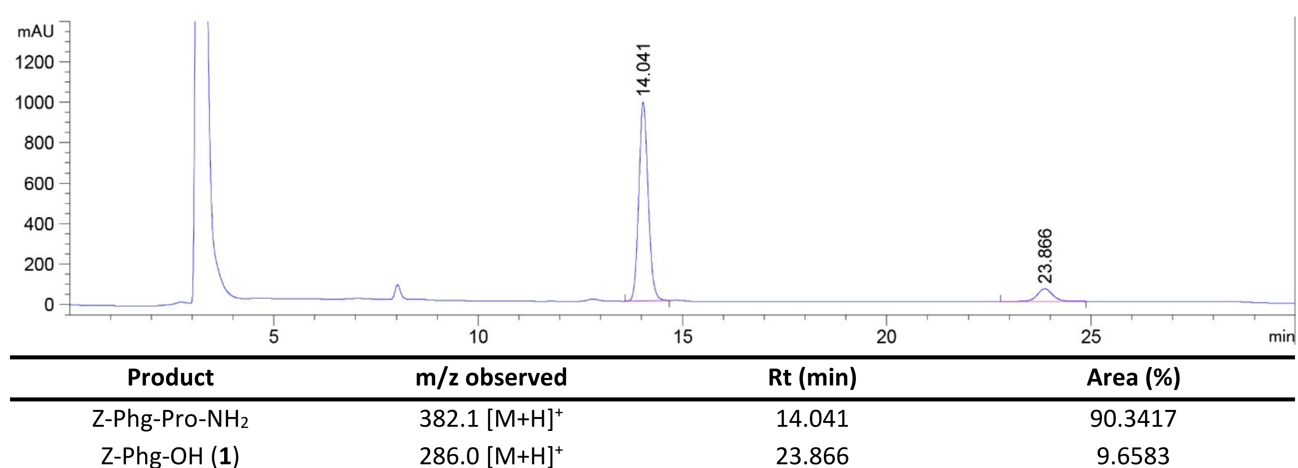
Product	m/z observed	Rt (min)	Area (%)
Z-Phg-Pro-NH$_2$	382.1 [M+H]$^+$	14.035	42.0193
Z-Phg-OH (**1**)	286.1 [M+H]$^+$	23.850	57.9807

Figure S6. Chromatogram of Z-Phg-Pro-NH$_2$ **(3)** at 220 nm (entry 2, Table S2).



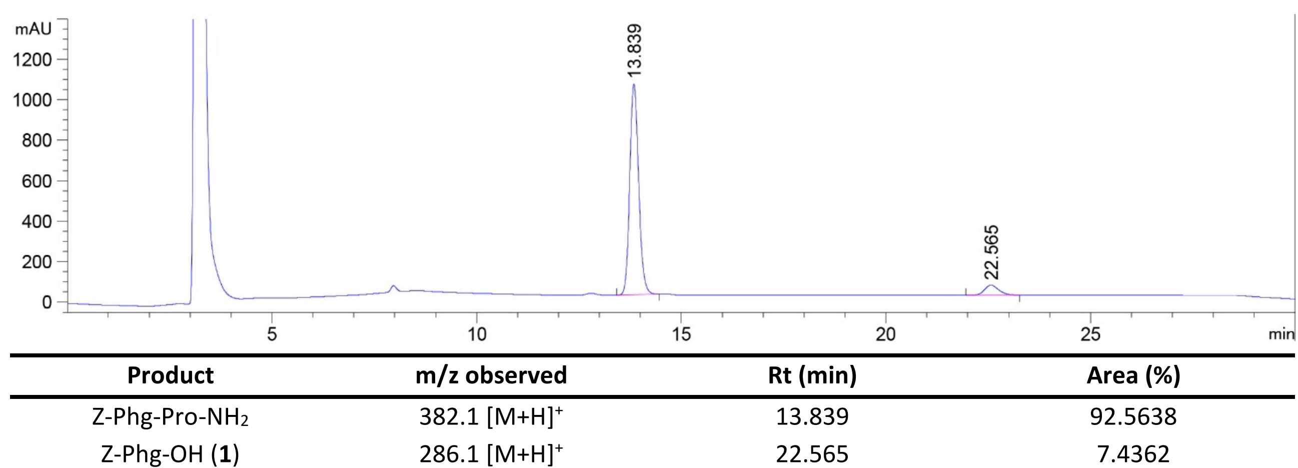
Product	m/z observed	Rt (min)	Area (%)
Z-Phg-Pro-NH_2	382.0 $[M+H]^+$	13.969	93.8632
Z-Phg-OH (**1**)	286.0 $[M+H]^+$	23.436	6.1368

Figure S7. Chromatogram of Z-Phg-Pro-NH_2 **(3)** at 220 nm (entry 3, Table S2).



Product	m/z observed	Rt (min)	Area (%)
Z-Phg-Pro-NH_2	382.1 $[M+H]^+$	14.041	90.3417
Z-Phg-OH (**1**)	286.0 $[M+H]^+$	23.866	9.6583

Figure S8. Chromatogram of Z-Phg-Pro-NH_2 **(3)** at 220 nm (entry 4, Table S2).



Product	m/z observed	Rt (min)	Area (%)
Z-Phg-Pro-NH_2	382.1 $[M+H]^+$	13.839	92.5638
Z-Phg-OH (**1**)	286.1 $[M+H]^+$	22.565	7.4362

Figure S9. Chromatogram of Z-Phg-Pro-NH_2 **(3)** at 220 nm (entry 5, Table S2).

3. Solvents screening and degree of racemization evaluation

General procedure

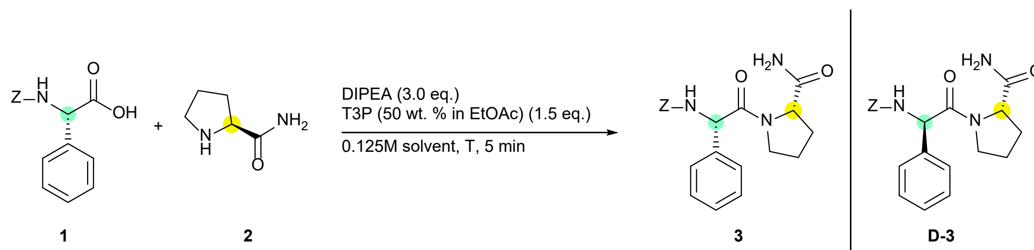


Figure S10. Synthesis of model peptide testing several green solvents.

In an oven-dried Schlenk purged under N_2 atmosphere, Z-Phg-OH (**1**) or Z-D-Phg-OH (29 mg, 0.1 mmol, 1 eq.) and H-Pro-NH_2 (**2**)(11 mg, 0.1 mmol, 1 eq.) were dissolved in the desired solvent (0.125M). Subsequently, DIPEA (52 µL, 0.3 mmol, 3 eq.) and T3P® (50 wt. % in EtOAc, 89 µL, 0.15 mmol, 1.5 eq.) were added at room temperature or 0°C following this order. The solution was stirred at room temperature for 5 minutes and the conversion was monitored by HPLC-MS analysis (Analysis Method 2 in Analytical methods section).

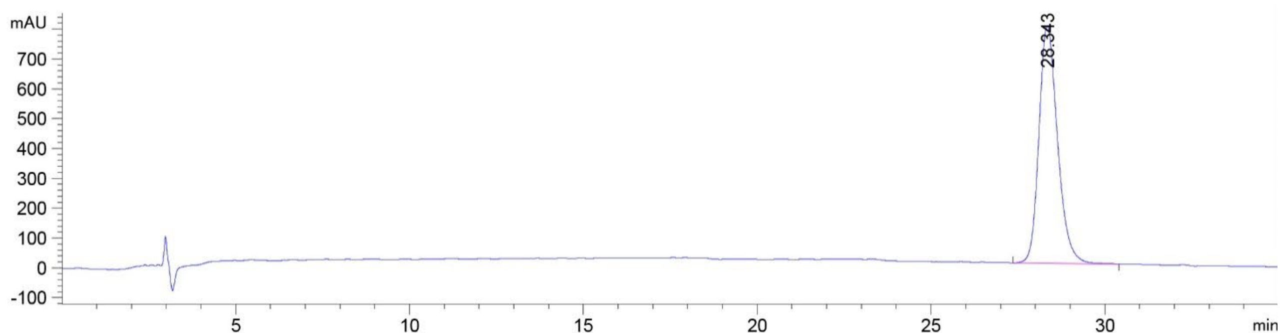
Table S3. Solvent screening

Entry[a]	Solvent	Temperature	3[d] (%)	(D)/(L+D) (%)
1	NBP	r.t.	56	0.6
2	NOP	r.t.	56	n.d.[b]
3	DMC	r.t.	93	0.2
4	GVL	r.t.	89	0.8
5	ACN	r.t.	96	0.2
6	THF	r.t.	93	0.1
7	2-MeTHF	r.t.	80	0.2
8	DCM	r.t.	98	0.2
9	*ⁱ*PrOAc	r.t.	69	1.1
10	EtOAc	r.t.	94	0.5
11	EtOAc	0°C to r.t.	96	0.3
12	DMF	r.t.	>99	0.5
13	DMF	0°C to r.t.	93	0.4
14[c]	DMF	rt	80	5.3

[a]The reactions were performed under the conditions used in entry 3, Table S1, and conversion evaluated after 5 minutes. [b]n.d. = not detected. [c]Pre-activation of the acid was performed by adding T3P® and DIPEA before Pro-NH_2. [d] Conversion obtained considering both 3 and D-3.

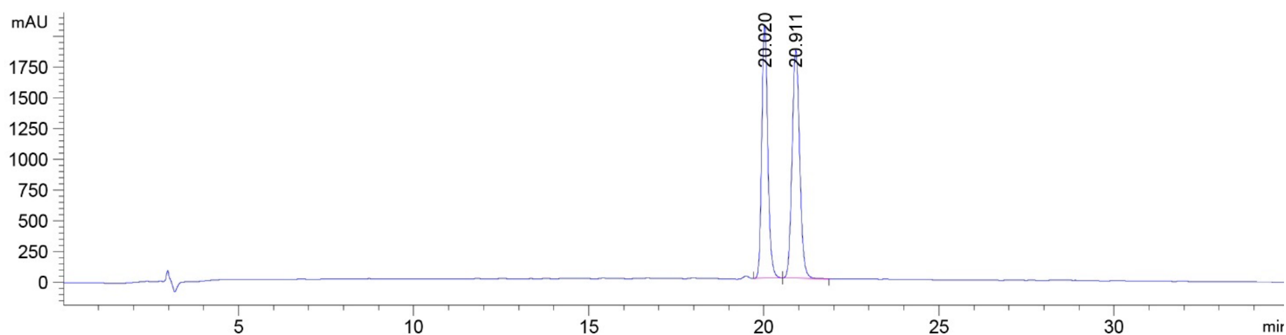
HPLC chromatograms

Peaks at 11.6 min, 5.4 min, 13.8, 8.8 min and 3.0 min are related to NBP, GVL, ^{i}PrOAc, EtOAc and DMF, respectively.



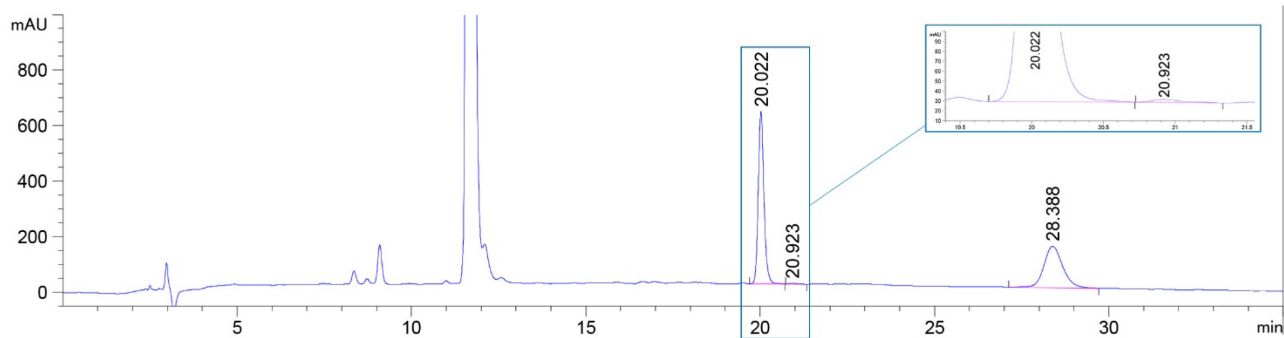
Product	m/z observed	Rt (min)
Z-Phg-OH	285.9 $[M+H]^+$	28.343

Figure S11. Chromatogram of Z-Phg-OH (**1**) at 210 nm (reference).



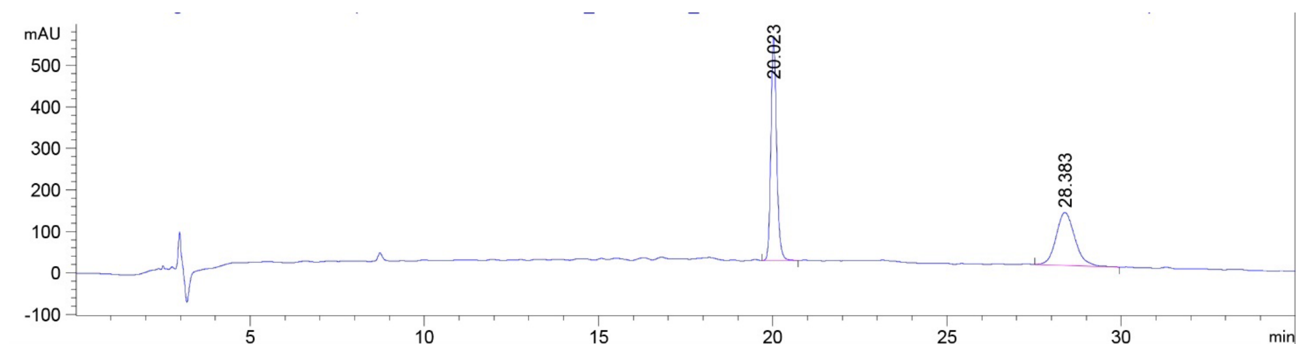
Product	m/z observed	Rt (min)	Area (%)
Z-Phg-Pro-NH_2	382.0 $[M+H]^+$	20.020	46.734
Z-D-Phg-Pro-NH_2	382.0 $[M+H]^+$	20.911	53.266

Figure S12. Chromatogram of the mixture Z-Phg-Pro-NH_2 (**3**) and Z-D-Phg-Pro-NH_2 (**D-3**) at 210 nm (reference).



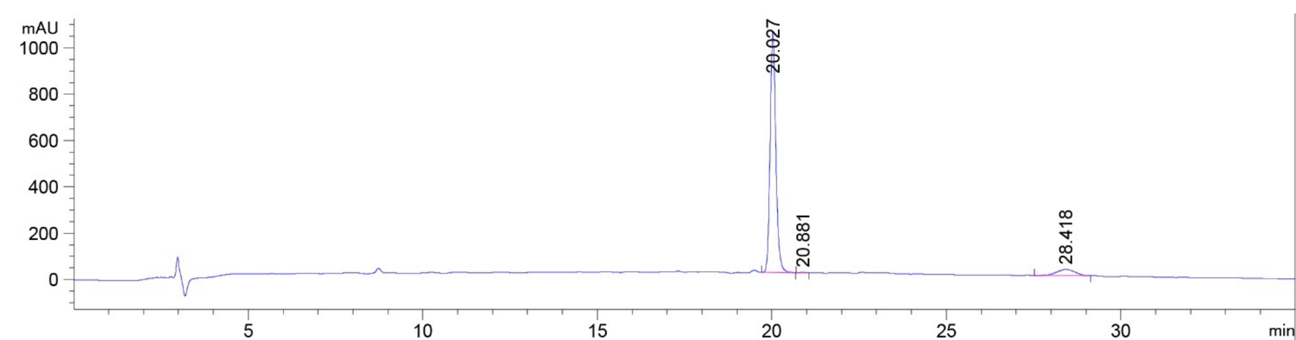
Product	m/z observed	Rt (min)	Area (%)
Z-Phg-Pro-NH_2	382.0 $[M+H]^+$	20.022	55.5696
Z-D-Phg-Pro-NH_2	403.9 $[M+Na]^+$	20.923	0.3562
Z-Phg-OH (**1**)	286.0 $[M+H]^+$	28.388	44.0742

Figure S13. Chromatogram of Z-Phg-Pro-NH_2 (**3**) in NBP at 210 nm (entry 1, Table S3).



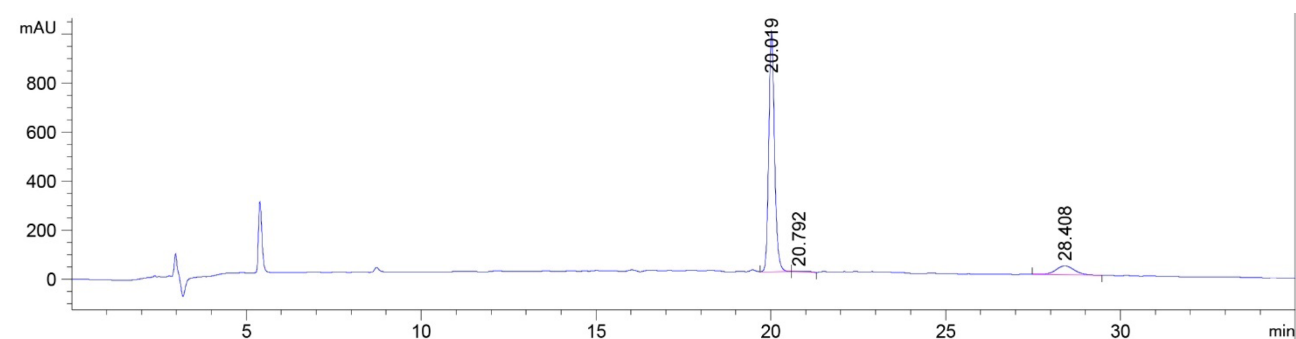
Product	m/z observed	Rt (min)	Area (%)
Z-Phg-Pro-NH_2	382.0 $[M+H]^+$	20.023	56.3748
Z-Phg-OH (**1**)	285.9 $[M+H]^+$	28.383	43.6252

Figure S14. Chromatogram of Z-Phg-Pro-NH_2 (**3**) in NOP at 210 nm (entry 2, Table S3).



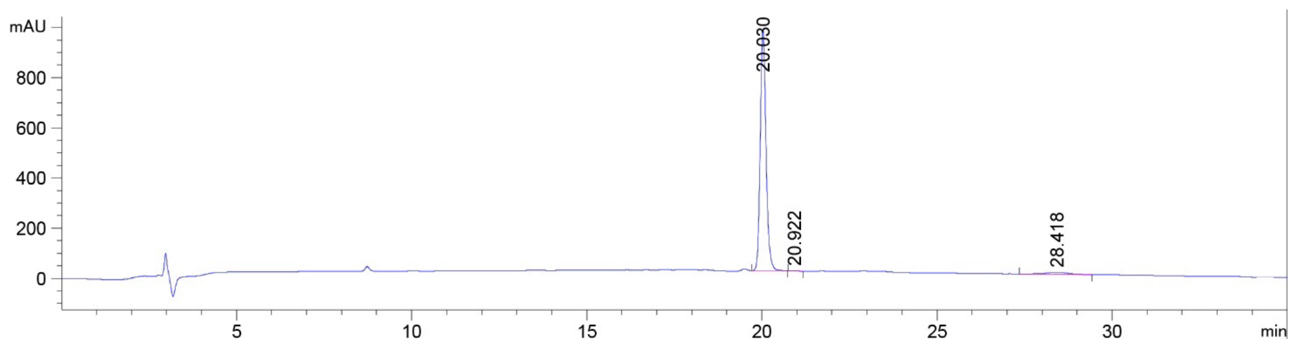
Product	m/z observed	Rt (min)	Area (%)
Z-Phg-Pro-NH_2	382.0 $[M+H]^+$	20.027	92.4101
Z-D-Phg-Pro-NH_2	403.9 $[M+Na]^+$	20.881	0.1601
Z-Phg-OH (**1**)	286.0 $[M+H]^+$	28.418	7.4298

Figure S15. Chromatogram of Z-Phg-Pro-NH_2 (**3**) in DMC at 210 nm (entry 3, Table S3).



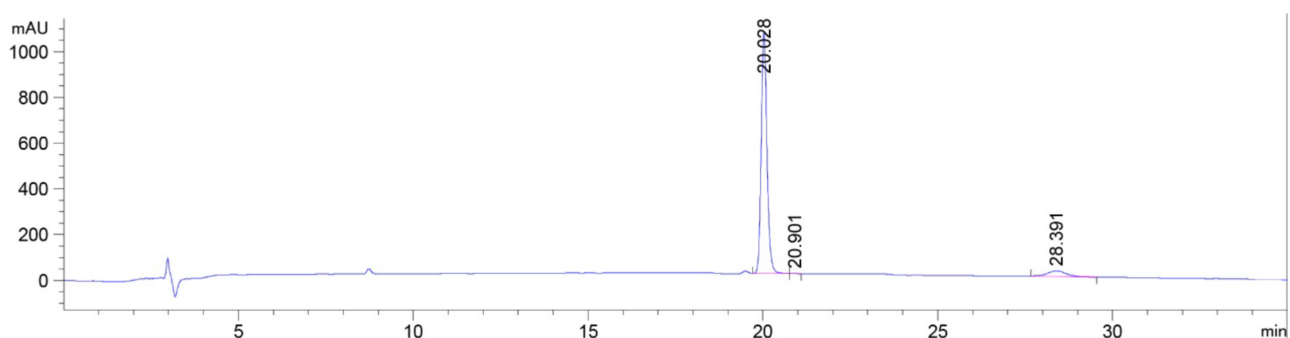
Product	m/z observed	Rt (min)	Area (%)
Z-Phg-Pro-NH_2	382.0 $[M+H]^+$	20.019	88.1827
Z-D-Phg-Pro-NH_2	382.0 $[M+H]^+$	20.792	0.6697
Z-Phg-OH (**1**)	285.8 $[M+H]^+$	28.408	11.1476

Figure S16. Chromatogram of Z-Phg-Pro-NH_2 (**3**) in GVL at 210 nm (entry 4, Table S3).



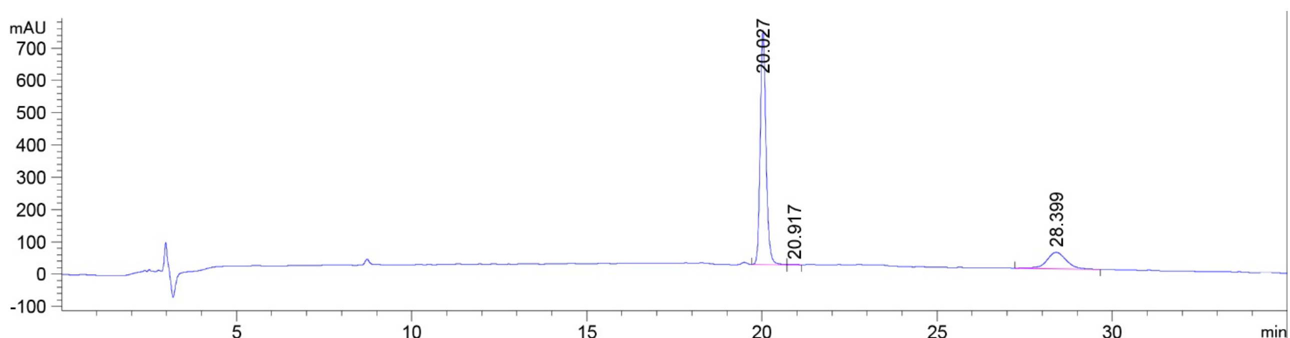
Product	m/z observed	Rt (min)	Area (%)
Z-Phg-Pro-NH_2	382.0 $[M+H]^+$	20.030	96.2462
Z-D-Phg-Pro-NH_2	382.0 $[M+H]^+$	20.922	0.1597
Z-Phg-OH (**1**)	286.0 $[M+H]^+$	28.418	3.5941

Figure S17. Chromatogram of Z-Phg-Pro-NH_2 (**3**) in ACN at 210 nm (entry 5, Table S3).



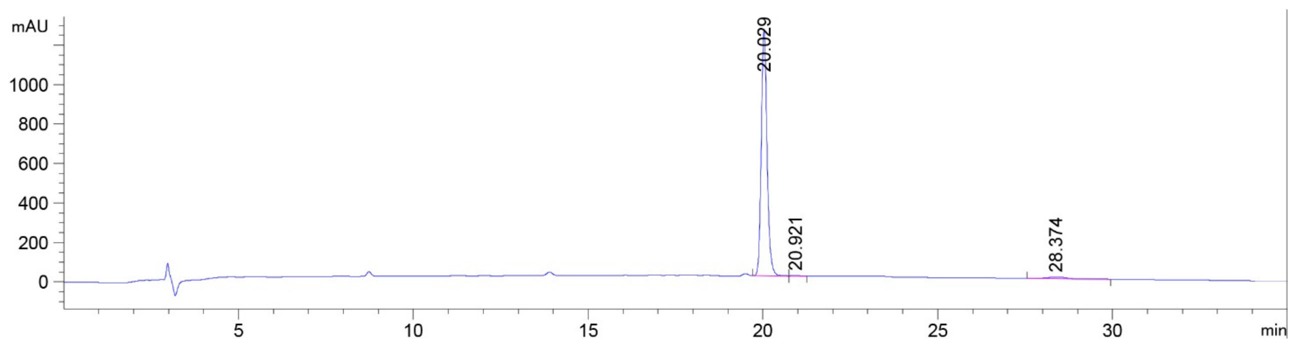
Product	m/z observed	Rt (min)	Area (%)
Z-Phg-Pro-NH_2	382.0 $[M+H]^+$	20.028	92.9681
Z-D-Phg-Pro-NH_2	381.9 $[M+H]^+$	20.901	0.0943
Z-Phg-OH (**1**)	285.9 $[M+H]^+$	28.391	6.9377

Figure S18. Chromatogram of Z-Phg-Pro-NH_2 (**3**) in THF at 210 nm (entry 6, Table S3).



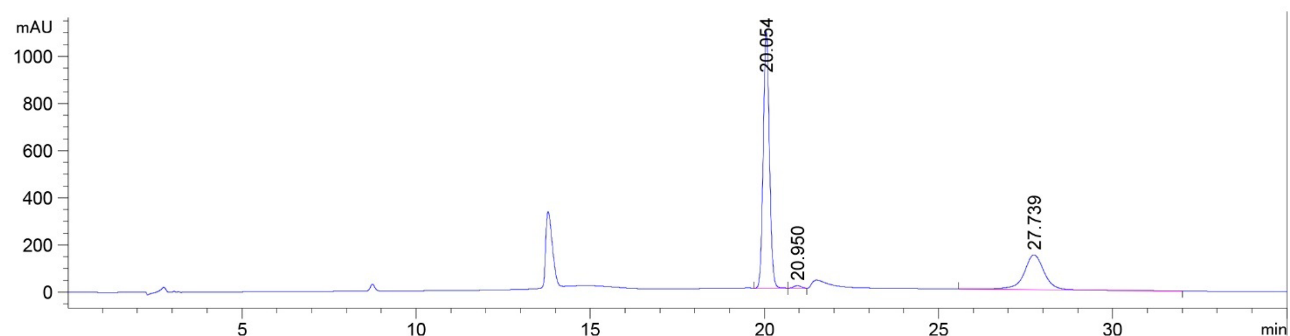
Product	m/z observed	Rt (min)	Area (%)
Z-Phg-Pro-NH_2	381.9 $[M+H]^+$	20.027	80.2050
Z-D-Phg-Pro-NH_2	381.9 $[M+H]^+$	20.917	0.1681
Z-Phg-OH (**1**)	285.9 $[M+H]^+$	28.399	19.6270

Figure S19. Chromatogram of Z-Phg-Pro-NH_2 (**3**) in 2-MeTHF at 210 nm (entry 7, Table S3).



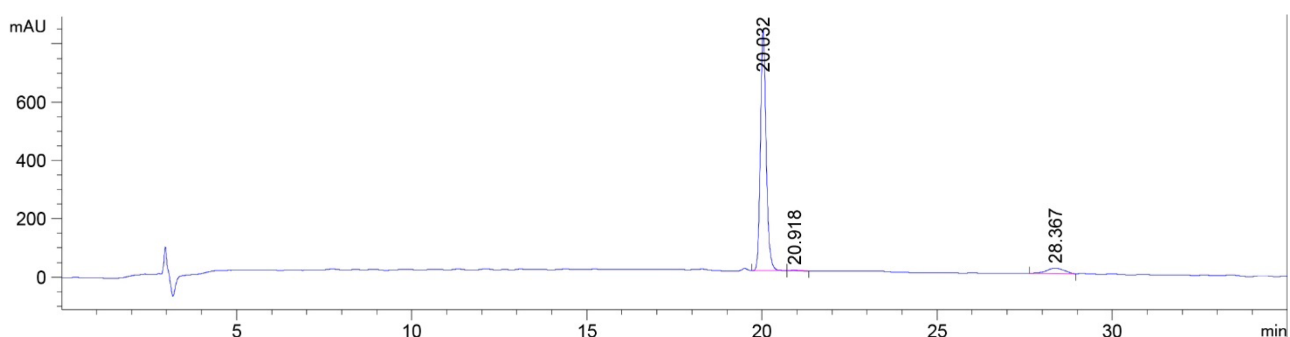
Product	m/z observed	Rt (min)	Area (%)
Z-Phg-Pro-NH_2	382.0 $[M+H]^+$	20.029	97.5774
Z-D-Phg-Pro-NH_2	382.0 $[M+H]^+$	20.921	0.2422
Z-Phg-OH (**1**)	286.0 $[M+H]^+$	28.374	2.1804

Figure S20. Chromatogram of Z-Phg-Pro-NH_2 (**3**) in DCM at 210 nm (entry 8, Table S3).



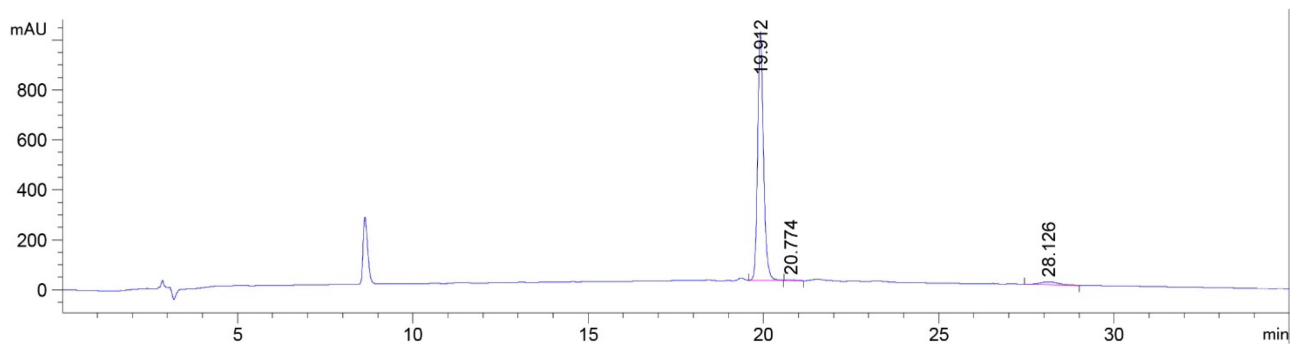
Product	m/z observed	Rt (min)	Area (%)
Z-Phg-Pro-NH_2	382.1 $[M+H]^+$	20.054	68.3147
Z-D-Phg-Pro-NH_2	382.1 $[M+H]^+$	20.950	0.7465
Z-Phg-OH (**1**)	286.1 $[M+H]^+$	27.739	30.9387

Figure S21. Chromatogram of Z-Phg-Pro-NH_2 (**3**) in ^{i}PrOAc at 210 nm (entry 9, Table S3).



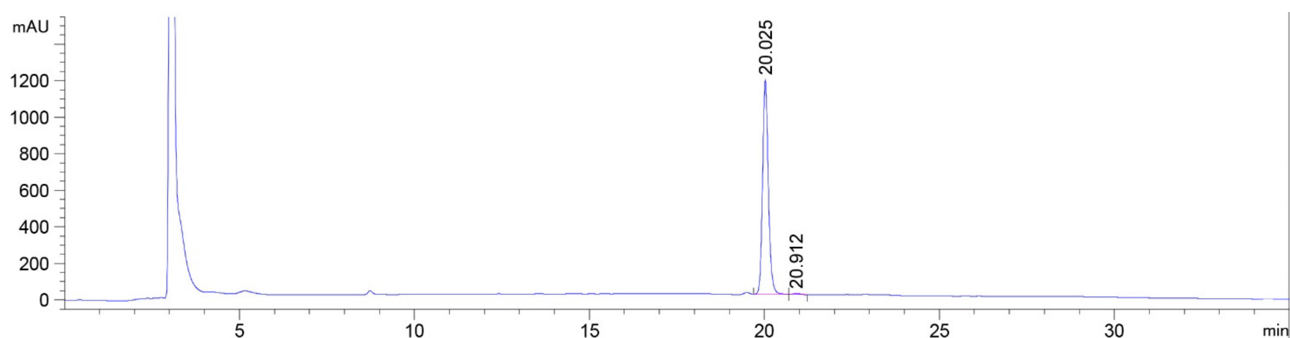
Product	m/z observed	Rt (min)	Area (%)
Z-Phg-Pro-NH_2	381.9 $[M+H]^+$	20.032	93.3116
Z-D-Phg-Pro-NH_2	382.0 $[M+H]^+$	20.918	0.4946
Z-Phg-OH (**1**)	285.9 $[M+H]^+$	28.367	6.1938

Figure S22. Chromatogram of Z-Phg-Pro-NH_2 (**3**) in EtOAc at rt at 210 nm (entry 10, Table S3).



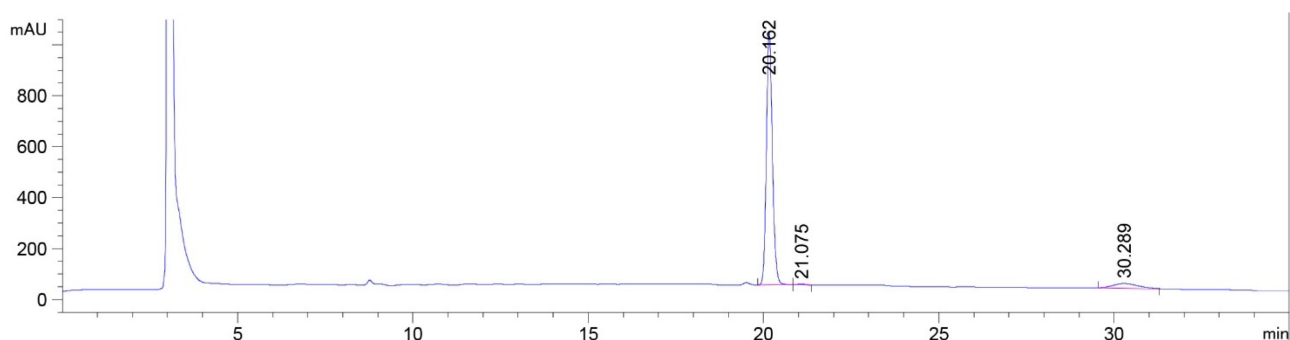
Product	m/z observed	Rt (min)	Area (%)
Z-Phg-Pro-NH_2	381.9 $[M+H]^+$	19.912	96.0499
Z-D-Phg-Pro-NH_2	404.0 $[M+Na]^+$	20.774	0.2934
Z-Phg-OH (**1**)	286.0$[M+H]^+$	28.126	3.6567

Figure S23. Chromatogram of Z-Phg-Pro-NH_2 (**3**) in EtOAc from 0°C to rt at 210 nm (entry 11, Table S3).



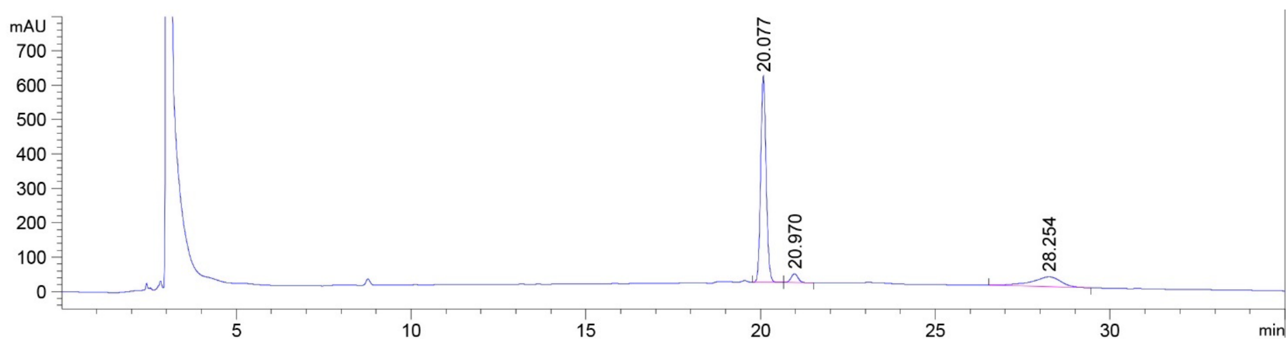
Product	m/z observed	Rt (min)	Area (%)
Z-Phg-Pro-NH_2	382.0 $[M+H]^+$	20.025	99.4600
Z-D-Phg-Pro-NH_2	381.9 $[M+H]^+$	20.912	0.5400

Figure S24. Chromatogram of Z-Phg-Pro-NH_2 (**3**) in DMF at rt at 210 nm (entry 12, Table S3).



Product	m/z observed	Rt (min)	Area (%)
Z-Phg-Pro-NH_2	382.1$[M+H]^+$	20.162	92.4526
Z-D-Phg-Pro-NH_2	382.1 $[M+H]^+$	21.075	0.3522
Z-Phg-OH (**1**)	286.0 $[M+H]^+$	30.289	7.1952

Figure S25. Chromatogram of Z-Phg-Pro-NH_2 (**3**) in DMF from 0°C to rt at 210 nm (entry 13, Table S3).



Product	m/z observed	Rt (min)	Area (%)
Z-Phg-Pro-NH_2	382.1 $[M+H]^+$	20.077	75.6027
Z-D-Phg-Pro-NH_2	382.1 $[M+H]^+$	20.970	4.2708
Z-Phg-OH (**1**)	286.0 $[M+H]^+$	28.254	20.1265

Figure S26. Chromatogram of Z-Phg-Pro-NH_2 (**3**) at 210 nm in DMF (Entry 14, Table S3).

4. Comparison between EtOAc and DMF using Oxyma Pure®/DIC.

General procedure

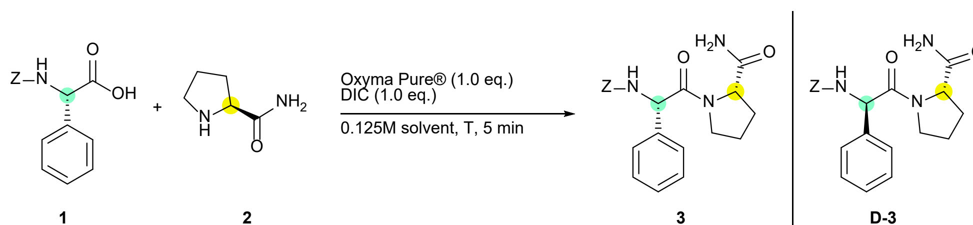


Figure S27. Synthesis of model peptide comparing DMF and EtOAc employing Oxyma Pure®/DIC.

Z-Phg-OH (**1**) (29 mg, 0.1 mmol, 1 eq.) was dissolved in a glass vial in DMF or EtOAc (0.125M). Subsequently, Oxyma Pure® (14 mg, 0.1 mmol, 1.0 eq.) and DIC (13 mg, 0.1 mmol, 1.0 eq.) were added at room temperature or 0°C following this order. After 3 minutes, H-Pro-NH_2 (**2**) (11 mg, 0.1 mmol, 1.0 eq.) was added and the solution was stirred at room temperature for 5 minutes. The conversion was monitored by HPLC-MS analysis (Analysis Method 2 in Analytical methods section).

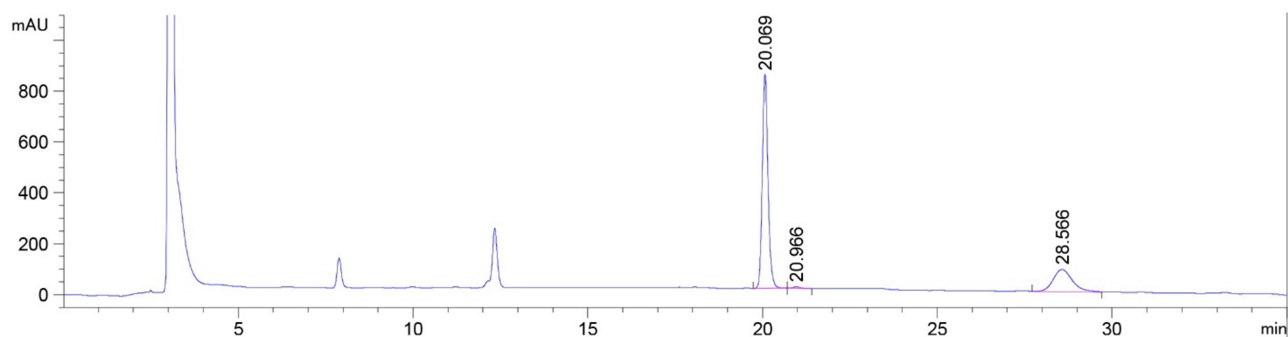
Table S4. Oxyma Pure®/DIC coupling in EtOAc and DMF

Entry[a]	Solvent	Temperature	3[c] (%)	(D)/(L+D) (%)
1	DMF	r.t.	74[b]	1.0
2	DMF	0°C to r.t.	69[b]	1.0
3	EtOAc	r.t.	97	1.0
4	EtOAc	0°C to r.t.	86	0.9

[a]The reactions were performed under the conditions used in entry 3, Table S1, and conversion evaluated after 5 minutes. [b]Conversion was 90-93% after 1h. [c] Conversion obtained considering both 3 and D-3.

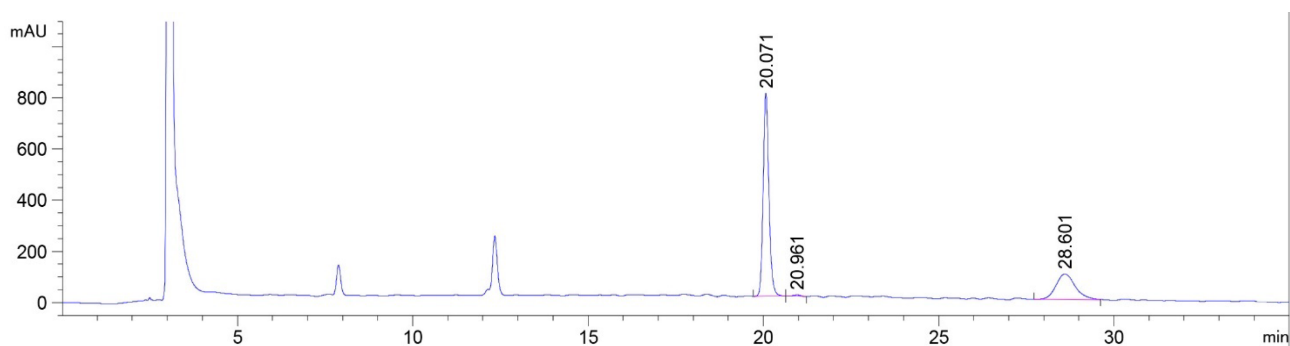
HPLC chromatograms

Peaks at 3.3, 7.9, 8.8 min and 12.4 min are related to DMF, DIC, EtOAc, and Oxyma Pure®, respectively.



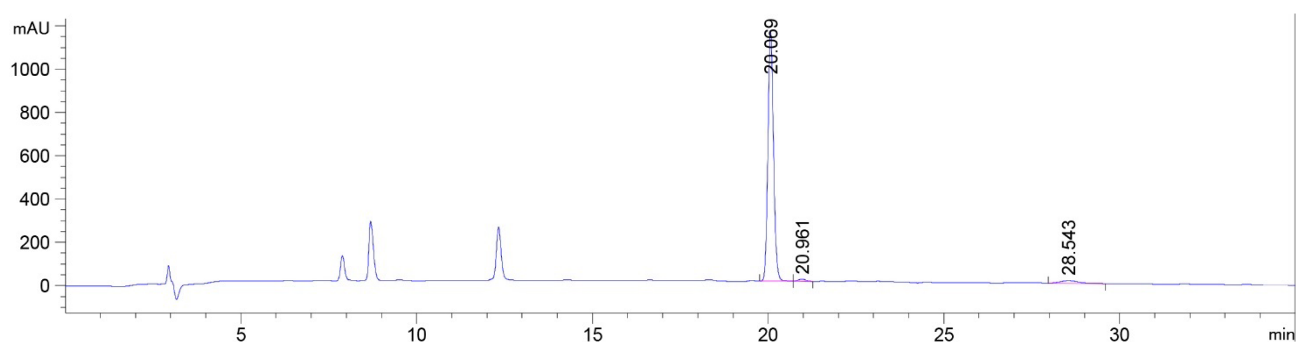
Product	m/z observed	Rt (min)	Area (%)
Z-Phg-Pro-NH_2	382.0 $[M+H]^+$	20.069	73.3257
Z-D-Phg-Pro-NH_2	382.8 $[M+H]^+$	20.966	0.7235
Z-Phg-OH (**1**)	285.9 $[M+H]^+$	28.566	25.9505

Figure S28. Chromatogram of Z-Phg-Pro-NH_2 (**3**) in DMF at rt at 210 nm (Entry 1, Table S4).



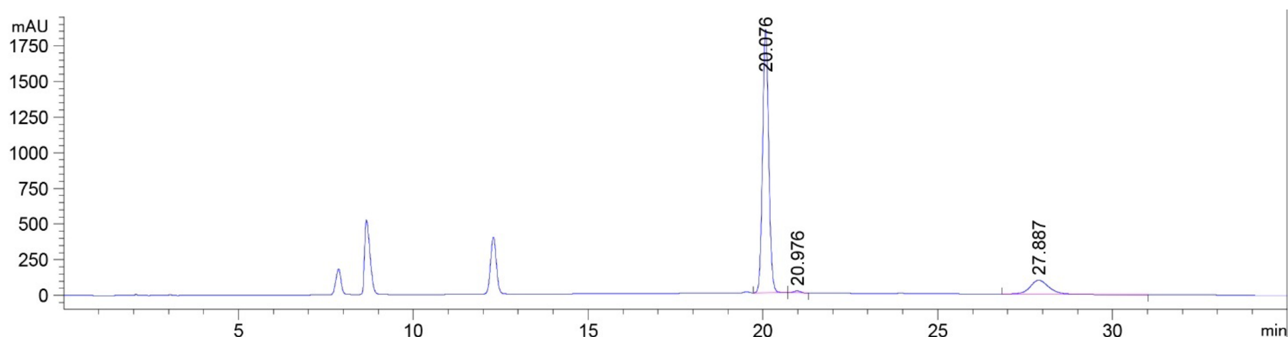
Product	m/z observed	Rt (min)	Area (%)
Z-Phg-Pro-NH_2	382.0 $[M+H]^+$	20.071	68.7144
Z-D-Phg-Pro-NH_2	382.0 $[M+H]^+$	20.961	0.7218
Z-Phg-OH (**1**)	286.0 $[M+H]^+$	28.601	30.5638

Figure S29. Chromatogram of Z-Phg-Pro-NH_2 (**3**) in DMF at 210 nm (Entry 2, Table S4).



Product	m/z observed	Rt (min)	Area (%)
Z-Phg-Pro-NH_2	382.0 $[M+H]^+$	20.069	95.6334
Z-D-Phg-Pro-NH_2	382.1 $[M+H]^+$	20.961	0.9792
Z-Phg-OH (**1**)	286.1 $[M+H]^+$	28.543	3.3874

Figure S30. Chromatogram of Z-Phg-Pro-NH_2 (**3**) in EtOAc at 210 nm (Entry 3, Table S4).



Product	m/z observed	Rt (min)	Area (%)
Z-Phg-Pro-NH_2	382.1 $[M+H]^+$	20.076	84.9116
Z-D-Phg-Pro-NH_2	382.1 $[M+H]^+$	20.976	0.7371
Z-Phg-OH (**1**)	286.1 $[M+H]^+$	27.887	14.3514

Figure S31. Chromatogram of Z-Phg-Pro-NH_2 (**3**) in EtOAc at 210 nm (Entry 4, Table S4).

5. Synthesis of Z-Phe-Leu-O^{t}Bu in gram scale

General method

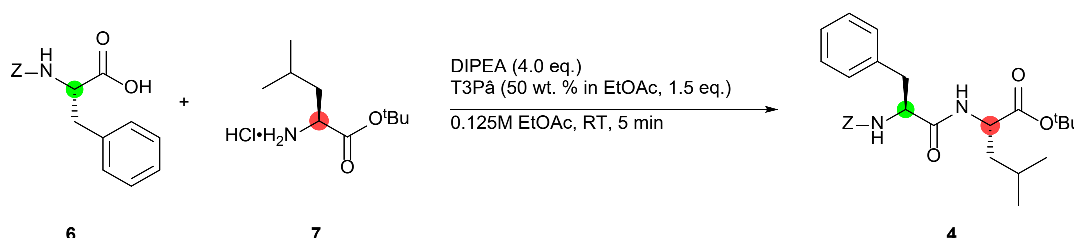
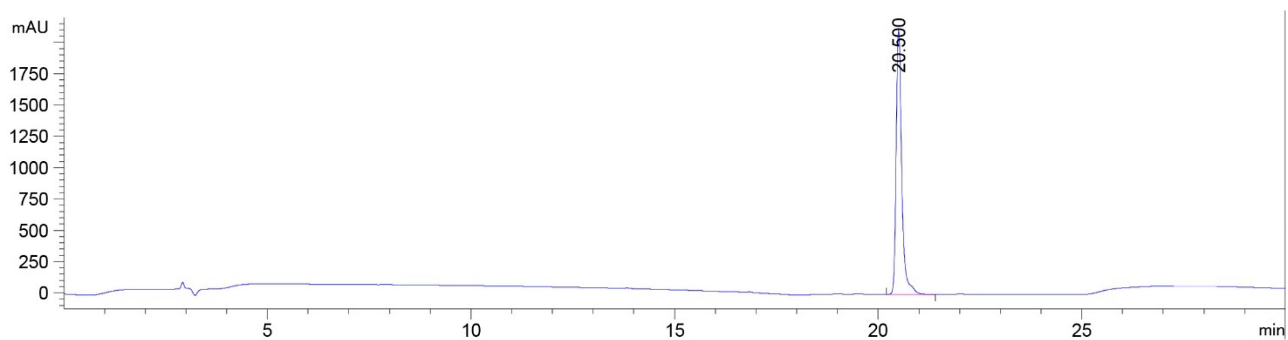


Figure S32. Synthesis of model peptide in gram scale.

An oven-dried, double-neck, 250 ml round-bottomed flask equipped with stirring bar Z-Phe-OH (**6**) (6.91 g, 23.1 mmol, 1.05 eq.), H_2N-Leu-O^{t}Bu hydrochloride (**7**) (4.92 g, 22 mmol, 1.0 eq.) and EtOAc (0.2M) were charged under N_2 atmosphere. Subsequently, DIPEA (15.3 mL, 88 mmol, 4.0 eq.) and T3P® (50 wt.% in EtOAc, 19.7 mL, 33 mmol, 1.5 eq.) were added following this order. The solution was stirred at room temperature for 5 minutes and the conversion was monitored by HPLC-MS analysis (Analysis method 3 in Analytical methods section). The solution was washed with H_2O (100 mL) and $NaHCO_{3(sat)}$ (100 mL). The organic phase was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. A white solid was obtained (Yield = 97%).

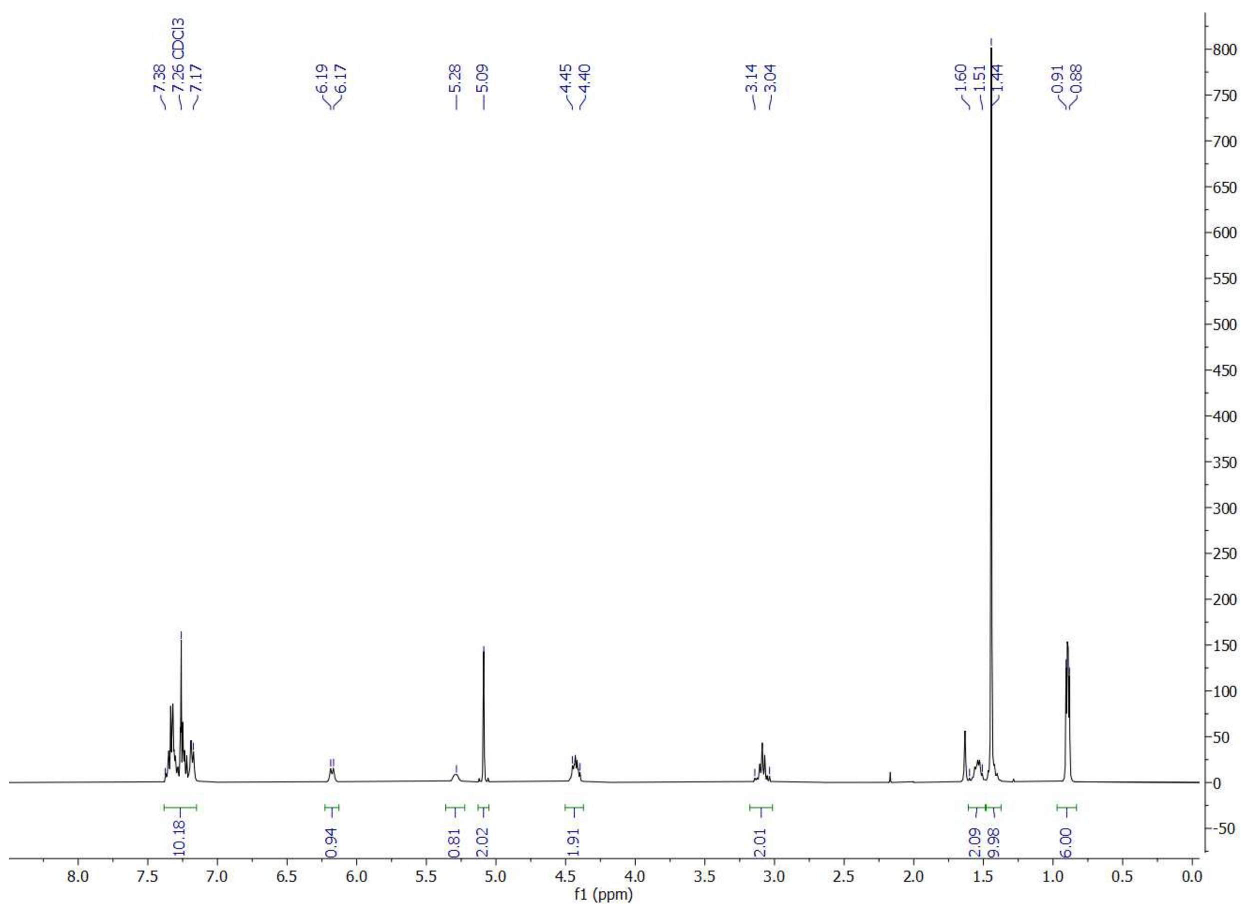
^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 7.38 – 7.17 (m, 10H), 6.18 (d, *J* = 8.2 Hz, 1H), 5.28 (s, 1H), 5.09 (s, 2H), 4.45 – 4.40 (m, 2H), 3.14 – 3.04 (m, 2H), 1.60 – 1.51 (m, 2H), 1.44 (s, 9H), 0.89 (td, *J* = 9.9 Hz, 6H).
^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) 171.68, 170.41, 136.35, 129.52, 128.75, 128.65, 128.30, 128.14, 127.14, 82.06, 67.15, 56.14, 51.58, 41.95, 38.50, 28.09, 24.91, 22.80, 22.24.

HPLC chromatograms and NMR spectra



Product	m/z observed	Rt (min)
Z-Phe-Leu-O^{t}Bu	469.2 $[M+H]^+$	20.500

Figure S33. Chromatogram of Z-Phe-Leu-O^{t}Bu (**4**) at 210 nm.



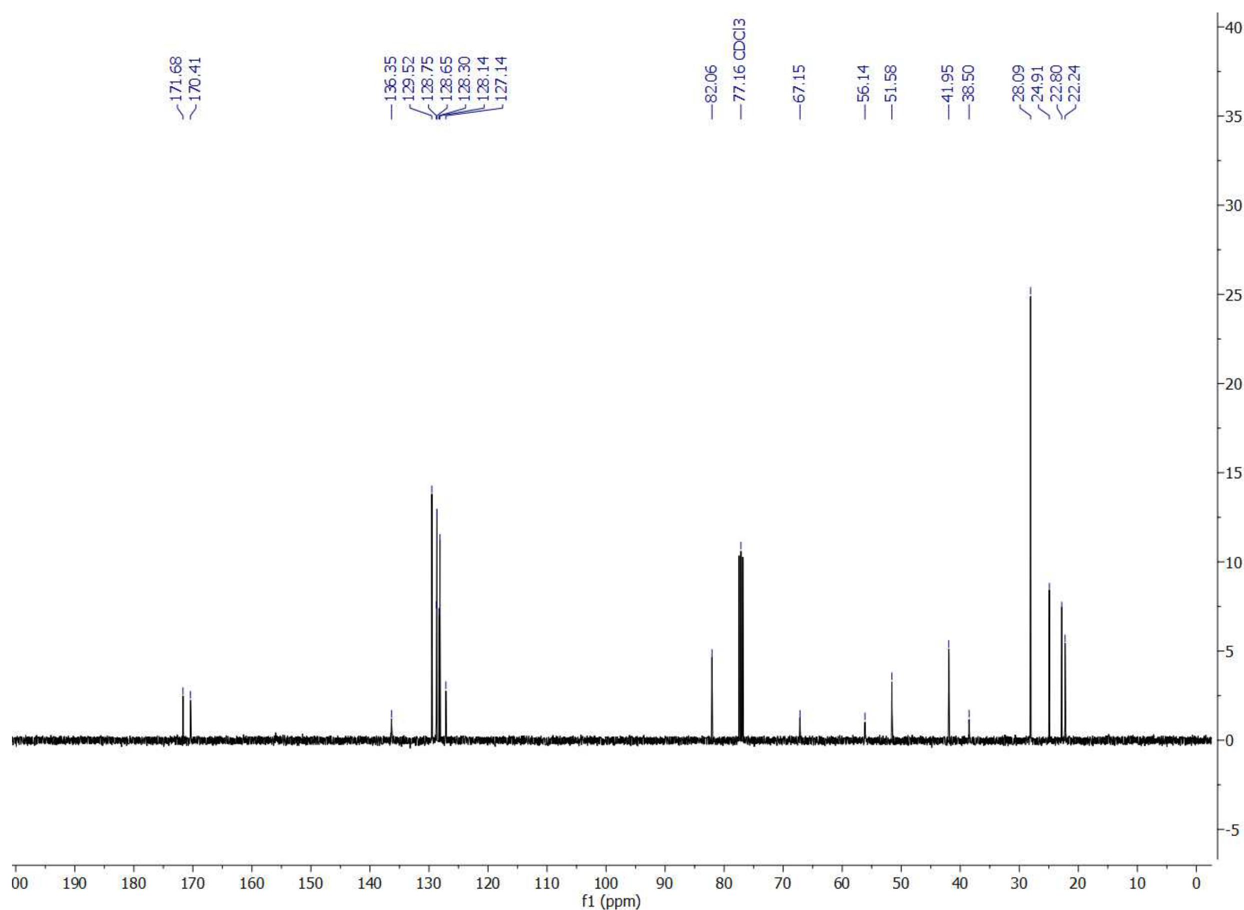


Figure S34. ^{1}H-NMR and ^{13}C-NMR of purified peptide Z-Phe-Leu-O^{t}Bu (**4**).

6. Dipeptides synthesis employing Aib

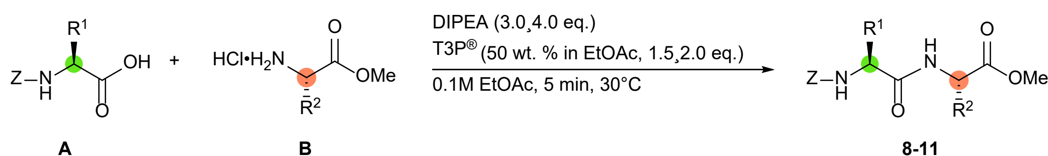


Figure S35. Aib coupling test.

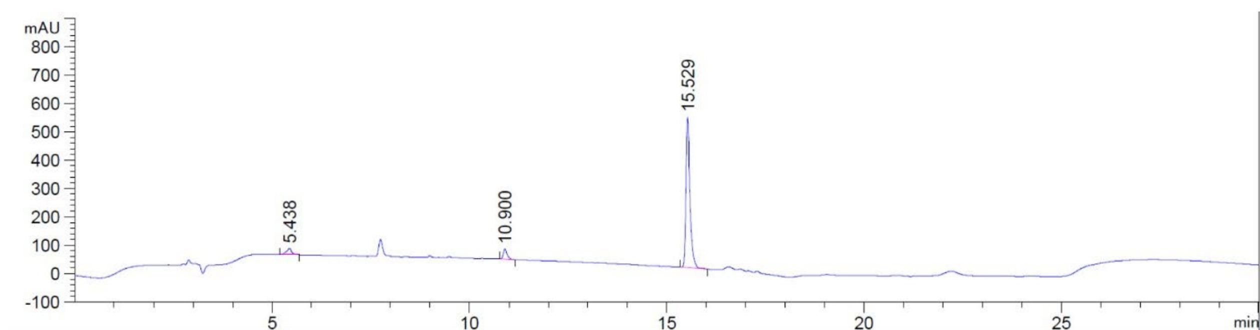
Following the procedure previously reported for a standard coupling reaction using EtOAc as solvent (see Par. 1, Supp. Info page S4), both Z-Aib-OH and H_2N-Aib-OMe hydrochloride were tested in the synthesis of different dipeptides to verify the effect of hinderance of Aib residues. The conversion was monitored by HPLC-MS analysis (Analysis Method 3 in Analytical methods section)

HPLC chromatograms

Table S5. Aib coupling tests.

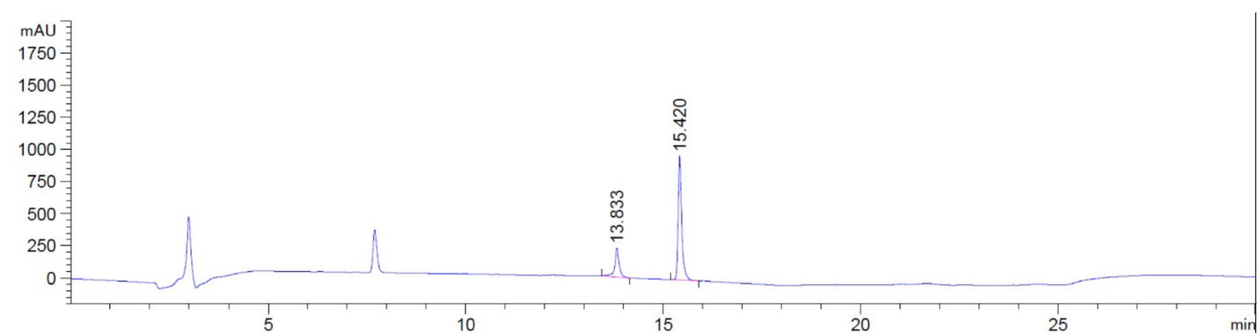
Entry[a]	A	B	Compound	DIPEA (eq)	T3P® (eq)	C (%)	Conditions
1	Aib	$ProNH_2$	**8**	4.0	2.0	92	
2	Aib	Phe	**9**	3.0	1.5	88	
3	Aib	Phe	**9**	4.0	2.0	95	
4	Aib	Phe	**9**	2.0+2.0	1.0+1.0	96	5 min (C = 71%), then add of the second portion of coupling reagents
5	Phe	Aib	**10**	4.0	2.0	78	
6	Aib	Aib	**11**	4.0	2.0	4	
7	Aib	Aib	**11**	4.0	2.0	4	EtOAc:DMSO 9:1
8[b]	Aib	Aib	**11**	4.0	2.0	3	After 5 min, HCl·H-L-O^tBu was added affording 92% of Z-Aib-L-O^tBu

[a]1.0 eq of DIPEA is added to desalt HCl·H_2N-Phe-OMe or HCl·H_2N-Aib-OMe. [b]2.0 eq of DIPEA are added to desalt HCl·H_2N-Aib-OMe and HCl·H_2N-Leu-O^tBu



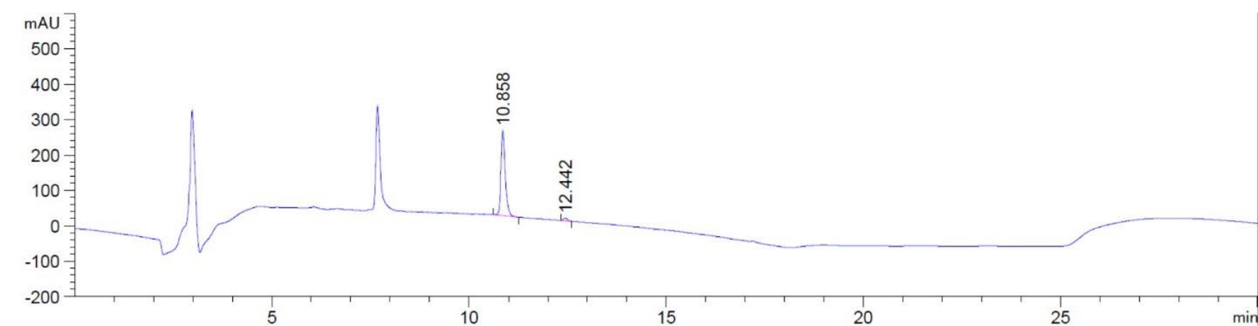
Product	m/z observed	Rt (min)	Area (%)
H_2N-Phe-OMe	180.1 $[M+H]^+$	5.438	4.5562
Z-Aib-OH	238.1 $[M+H]^+$	10.900	5.7105
Z-Aib-Phe-OMe	399.1 $[M+H]^+$	15.529	89.7333

Figure S36. Chromatogram at 210 nm of Z-Aib-Phe-OMe (**9**) (entry 4, Table S5).



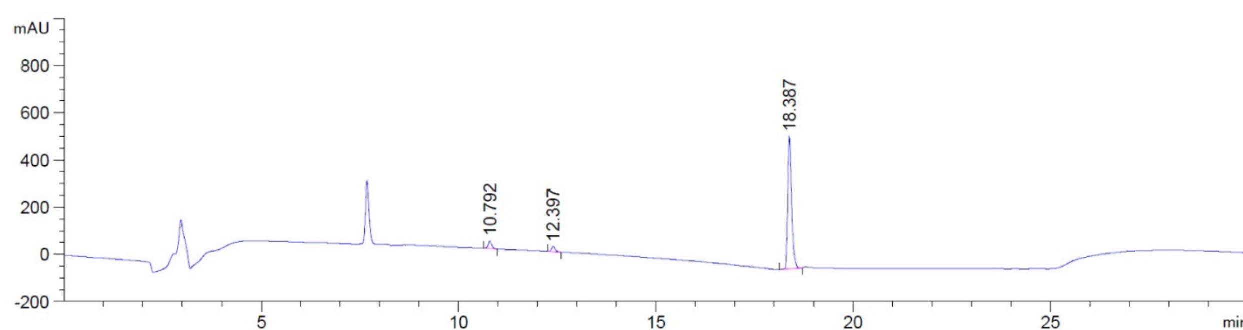
Product	m/z observed	Rt (min)	Area (%)
Z-Phe-OH	300.0 $[M+H]^+$	13.833	21.6119
Z-Phe-Aib-OMe	399.1 $[M+H]^+$	15.420	78.3881

Figure S37. Chromatogram at 210 nm of Z-Phe-Aib-OMe (**10**) (entry 5, Table S5).



Product	m/z observed	Rt (min)	Area (%)
Z-Aib-OH	237.9 [M+H]⁺	10.858	97.0251
Z-Aib-Aib-OMe	337.0 [M+H]⁺	12.442	2.9749

Figure S38. Chromatogram at 210 nm of Z-Aib-Aib-OMe (**11**) (entry 6, Table S5).



Product	m/z observed	Rt (min)	Area (%)
Z-Aib-OH	238.1 [M+H]⁺	10.792	4.7543
Z-Aib-Aib-OMe	337.1 [M+H]⁺	12.397	3.3474
Z-Aib-Leu-O^{t}Bu	407.2 [M+H]⁺	18.387	91.8982

Figure S39. Chromatogram at 210 nm of Z-Aib-Aib-OMe (**11**) (entry 8, Table S5).

7. Conditions screening for Z removal

7.a Calculation of Relative Response Factor (RRF)

The RRF was calculated using the following formula[1,2]:

$$RRF = \frac{HPLC\ Area_B}{HPLC\ Area_A} \cdot \frac{I_A}{I_B}$$

Where I is the integration obtained by quantitative ^{1}H-NMR (qNMR):

- relaxion delay = 60 s;
- acquisition time = 4 s;
- number of scans for 400 MHz = 12.

The molar ratio was calculated by qNMR through the integration of the peaks corresponding to the hydrogens in β-position for phenylalanine in both H-Phe-Leu-O^{t}Bu **A** (dd at 2.72 ppm) and Z-Phe-Leu-O^{t}Bu **B** (multiplet at 3.08 ppm).

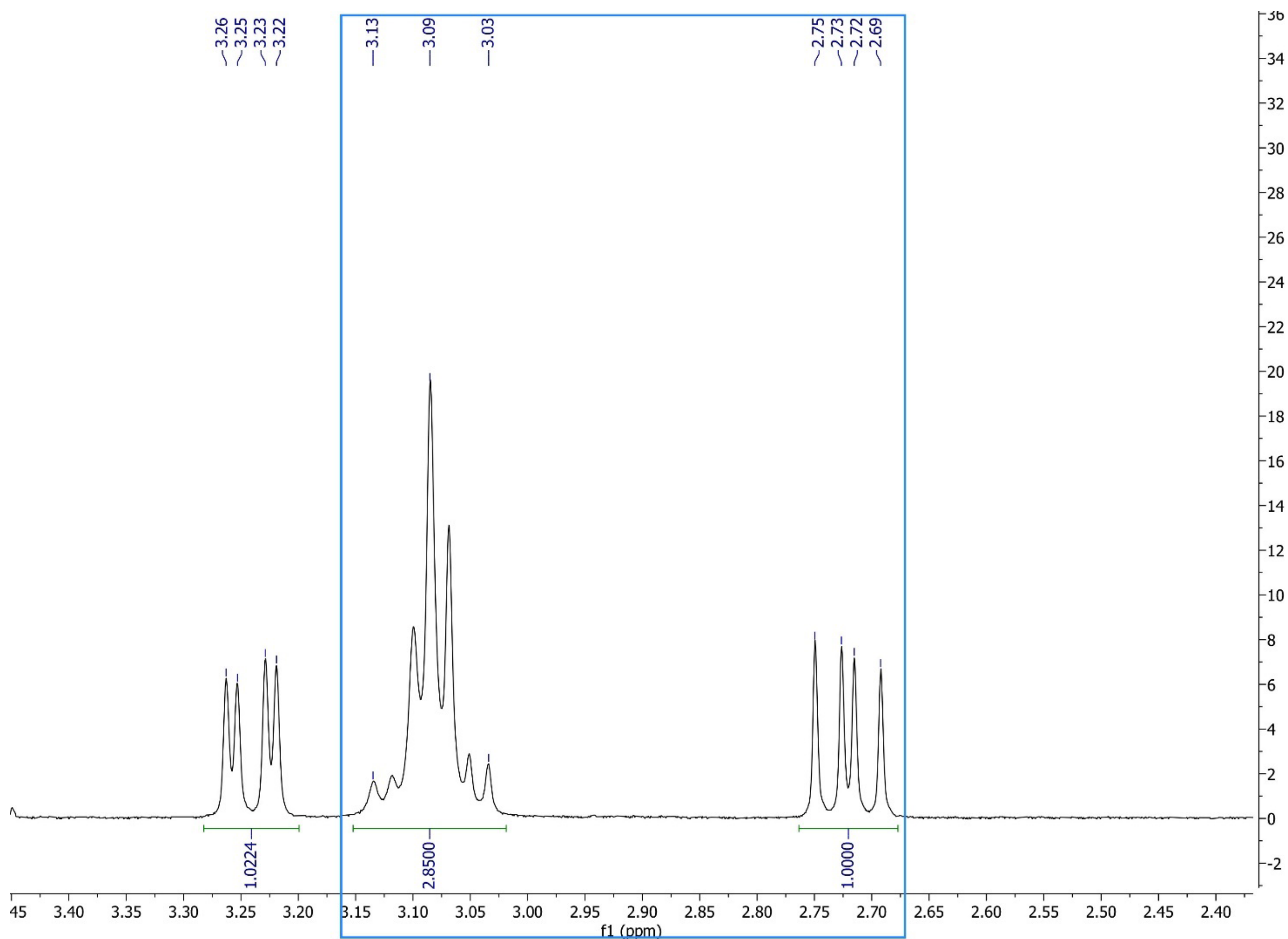
For HPLC separation, analytical method 3 was employed (fully elucidated in analytical methods section).

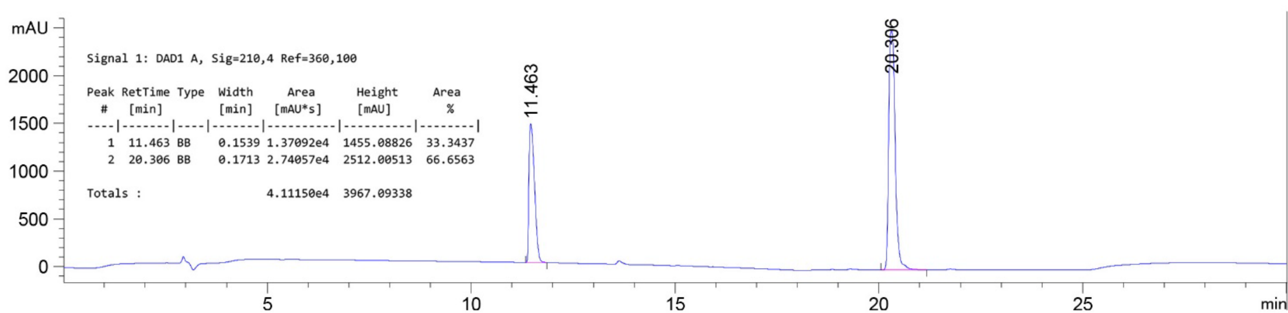
Table S6. RRF calculation. The experiments were repeated three times.

Sample	Molar ratio I_A/I_B	RRF at 210 nm
1	1.000/1.425	1.403
2	1.000/0.7303	1.772
3	1.000/0.3993	1.857
Average Value		1.677

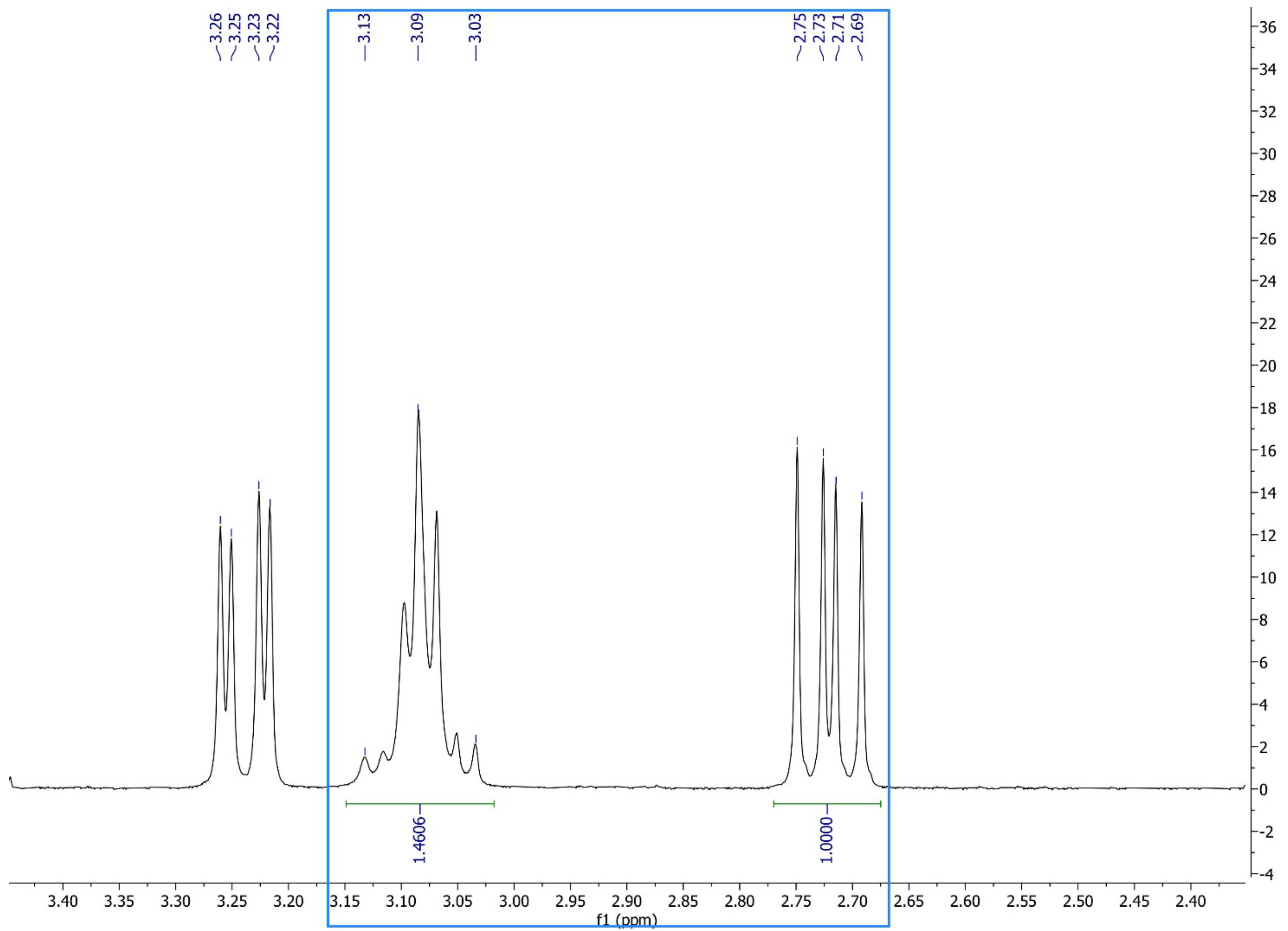
NMR spectra and HPLC chromatograms for RRF calculations

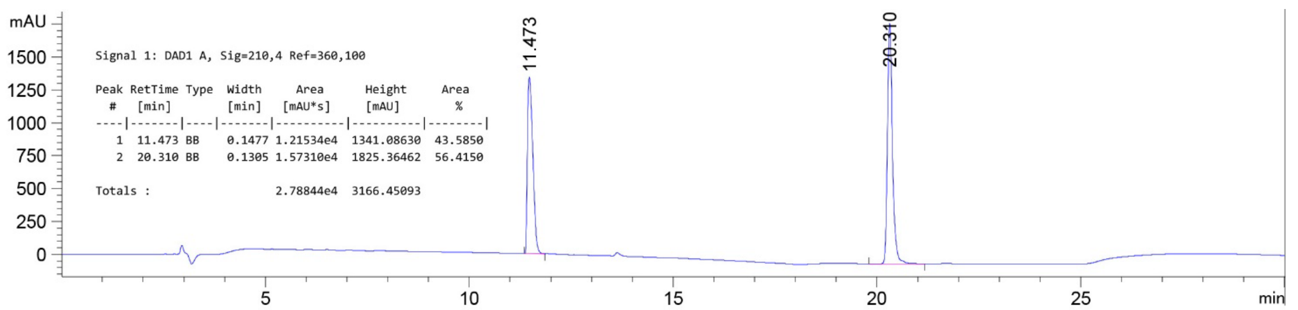
Sample 1: Molar ratio: I_A/I_B = 1.000/1.425





Sample 2: Molar ratio: I_A/I_B = 1.000/0.7303

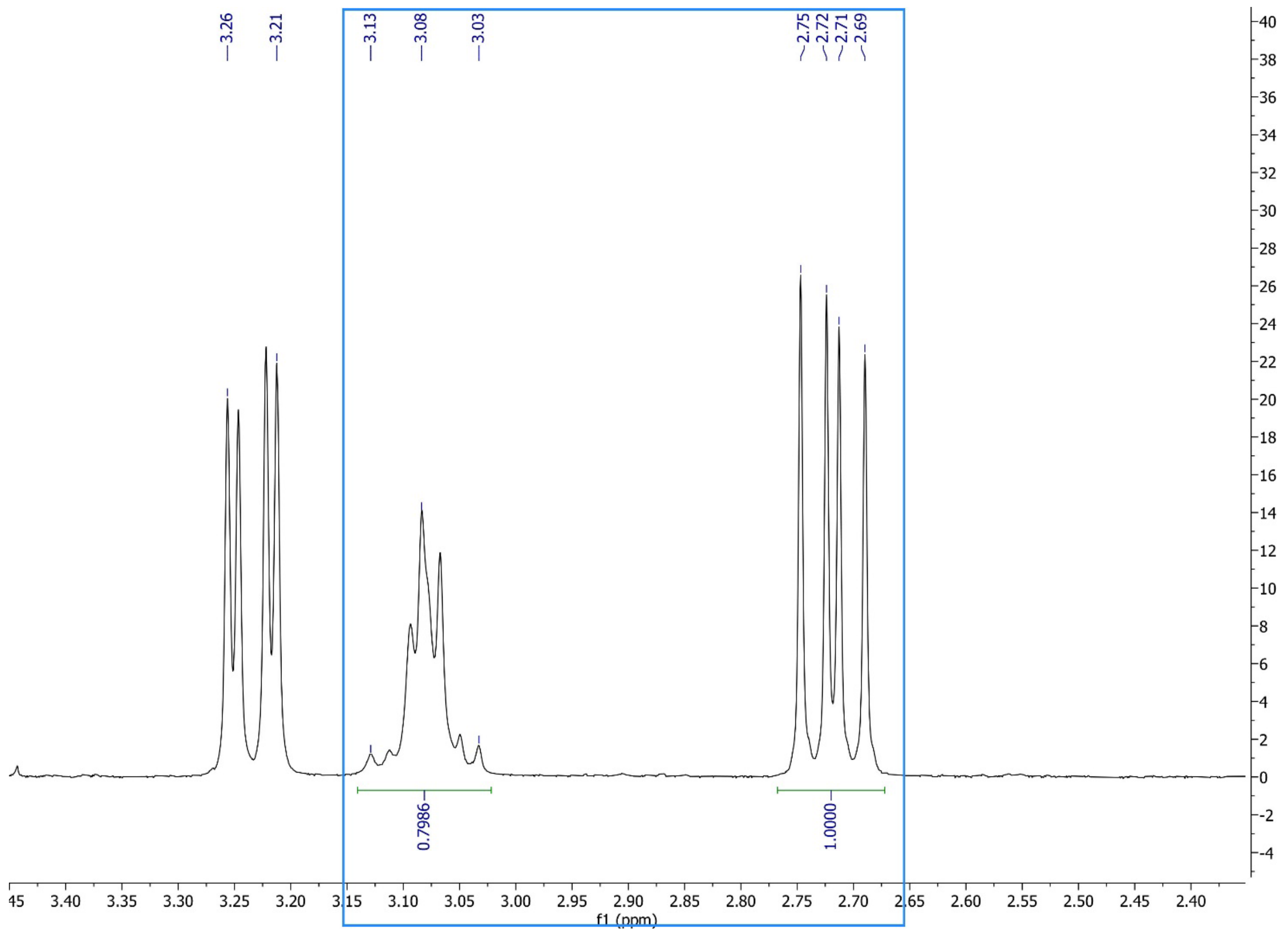


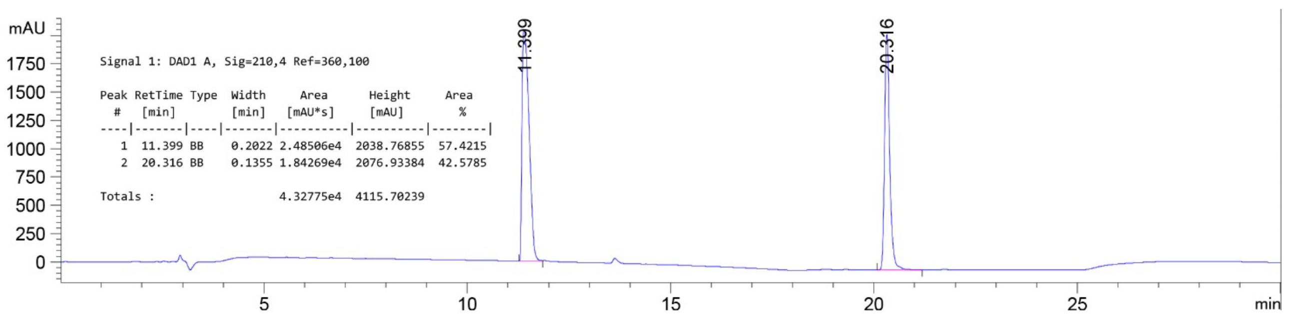

Signal 1: DAD1 A, Sig=210,4 Ref=360,100

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	11.473	BB	0.1477	1.21534e4	1341.08630	43.5850
2	20.310	BB	0.1305	1.57310e4	1825.36462	56.4150

Totals : 2.78844e4 3166.45093

Sample 3: Molar ratio: I_A/I_B = 1.000/0.3993





Signal 1: DAD1 A, Sig=210,4 Ref=360,100

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	11.399	BB	0.2022	2.48506e4	2038.76855	57.4215
2	20.316	BB	0.1355	1.84269e4	2076.93384	42.5785
Totals :				4.32775e4	4115.70239	

7.b Z removal by Method A

General procedure

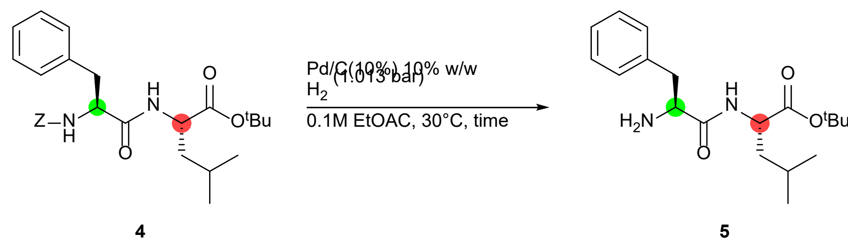


Figure S40. Removal of Z group by Method A.

In an oven-dried flask purged under N_2 atmosphere, Z-Phe-Leu-O^{t}Bu (**4**) (200 mg, 0.427 mmol, 1.0 eq.) and dry Pd/C$_{(10\%)}$ (10% w/w, 4.4 mol%) were added in EtOAc (0.1M). Subsequently, DIPEA, T3P® (50 wt. % in EtOAc), T3P® (50 wt. % in EtOAc)-H_2O (1.0 eq.-1.0 eq.), DIPEA-HCl (1.0 eq.-1.0 eq.) or none were added. The atmosphere was switched from nitrogen to H_2 gas (balloon), the reaction was stirred at 30°C and the conversion was monitored by HPLC-MS analysis (Analysis method 3 in Analytical methods section).

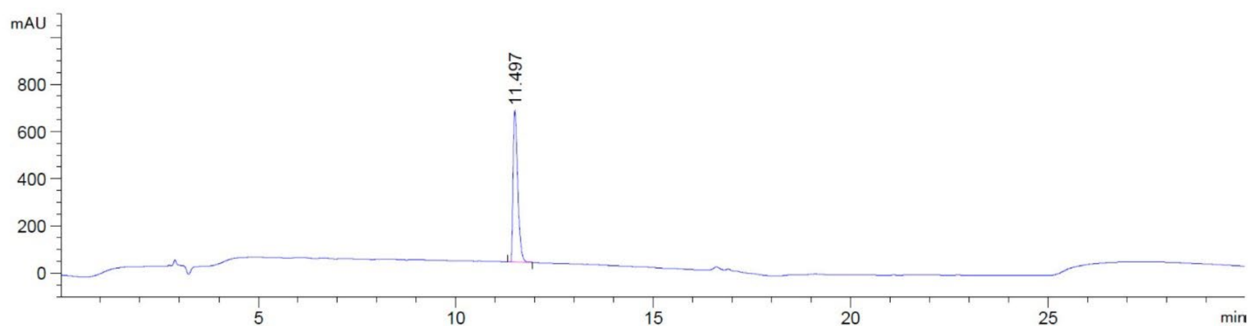
HPLC chromatograms

Chromatogram related to Z-Phe-Leu-O^{t}Bu (**4**, rt 20.5 min) is reported in the Par. 5 (Supp. Info page S16) (ESI) as reference.

Table S7. Screening of conditions for Z-F-L-O^{t}Bu **4** deprotection.

Entry	Contaminant (equiv)	Method	Time (min)	5 (%)
1	/	A[a]	300	>99
2	/	A	180	>99
3	DIPEA (0.1)	A	60	>99
4	DIPEA (3)	A	30	>99
5	T3P®:H₂O (1,5)	A	240	>99
6	DIPEA:T3P® (1.5/3)	A	120	>99
7	DIPEA:HCl (3)	A	240	>99

[a] Reaction performed with Pd/C$_{(10\%)}$ 10% w/w not previously dried under vacuum



Product	m/z observed	Rt (min)
H-Phe-Leu-O^{t}Bu	335.2 $[M+H]^+$	11.497

Figure S41. Chromatogram at 210 nm of H-Phe-Leu-O^{t}Bu (**5**) (entry 3, Table S7).

7.c Cbz removal by Method B

General procedure

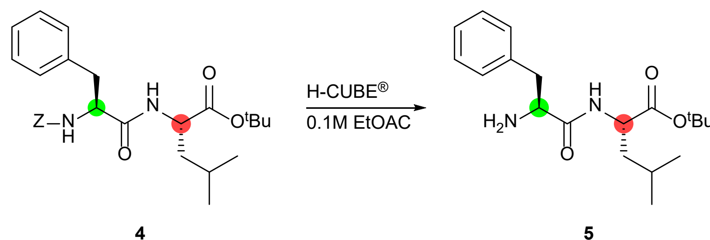


Figure S42. Removal of Z group by Method B.

Z-Phe-Leu-O^{t}Bu (**4**) (200 mg, 0.427 mmol, 1.0 eq) was dissolved in EtOAc (0.1M) and DIPEA, T3P® (50 wt. % in EtOAc), T3P® (50 wt. % in EtOAc)-H_2O (1.0 eq.-1.0 eq.) or none were added. The solution was flowed at 1 mL/min through the catalyst-packed cartridge (CatCart® - filled with Pd/$C_{(10\%)}$)* at 1 bar pressure, at 60°C and collecting 16 or 18 mL. The conversion was monitored by HPLC-MS analysis (Analysis method 3 in Analytical methods section). Finally, H-CUBE® was washed by ^{i}PrOH for 10 minutes to clean the system.

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* Cartridge dimensions: 0.4 cm (internal diameter) X 3 cm (length) = 0.377 mL total volume
Cartridge content: 40 mg of Pd/C10% corresponding to 4 mg of Pd (M.W. 106) = 0.0377 mmol of Pd
Pd/substrate ratio = 0.0377/0.427 = 8.8% mol

Calculation of local catalyst/substate concentration

We considered that the cartridge could be filled during reaction ½ by the catalyst and ½ by the substrate (rough evaluation considering the density of Pd/C catalyst)
Content of substrate 0.1 M solution in ½ cell volume = 0.0188 mmol (8.8 mg of dipeptide F.W. 469)
Catalyst/substrate ratio in the cartridge (mol/mol) = 0.0377 mmol Pd/0.0188 mmol substrate = 2/1
Catalyst/substrate ratio in the cartridge (w/w) = 4 mg/ 8.8 mg = 45% w/w

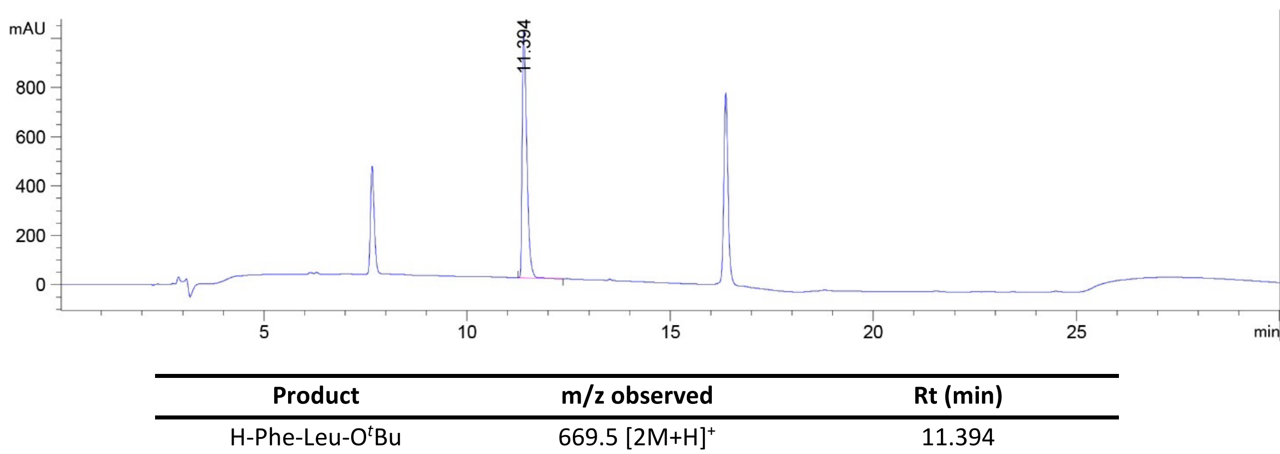
HPLC chromatograms

Chromatogram related to Z-Phe-Leu-O^{t}Bu (**4**, rt 20.5 min) is reported in the Par. 5 (Supp. Info page S16) (ESI) as reference. Peaks at 7.7 min and 16.4 min are related to EtOAc and Toluene, respectively.

Table S8. Screening of conditions for Z-F-L-O^{t}Bu **4** deprotection.

Entry	Contaminant (equiv)	Method	Time (min)	5 (%)
1	/	B [a]	18	>99
2	DIPEA (0.1)	B	16	>99
3	DIPEA (1)	B	18	>99
4	DIPEA:T3P® (1.5/3)	B	18	>99

[a]H-Cube setup generated 6 mL of death volume solvent and complete recovery of **5** was obtained by collecting solution in the following 18 minutes.



Product	m/z observed	Rt (min)
H-Phe-Leu-O^{t}Bu	669.5 $[2M+H]^+$	11.394

Figure S43. Chromatogram at 210 nm of H-Phe-Leu-O^{t}Bu (**5**) (Entry 2, Table S8).

8. Synthesis of fully protected Leu-Enkephalin in solution-phase by Method A$_{cont}$

General procedure

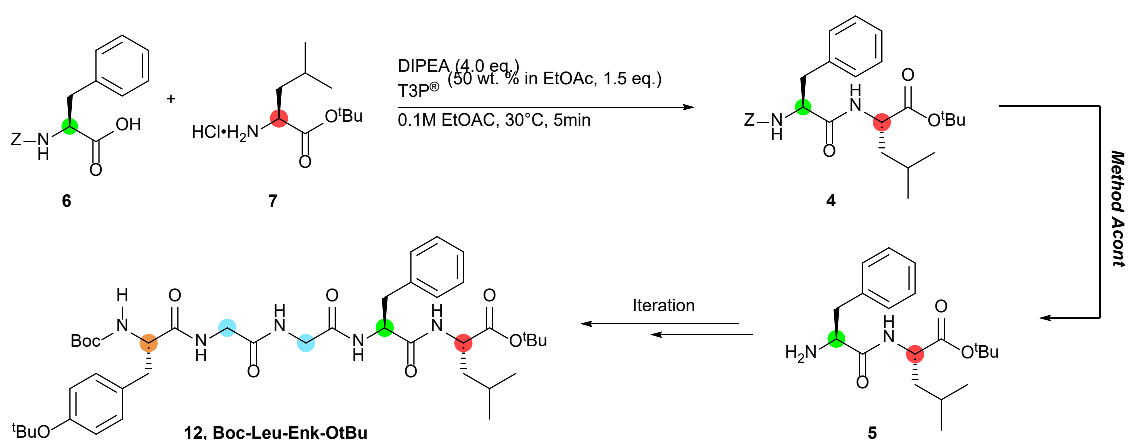


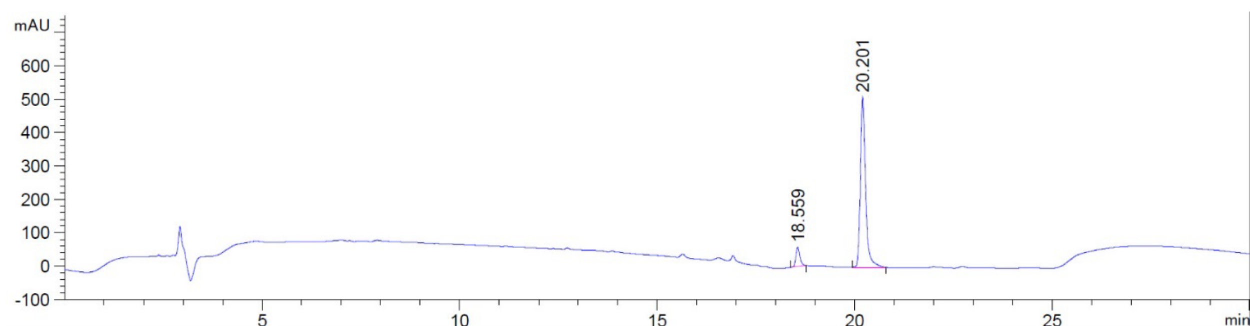
Figure S44. Synthesis of fully protected Leu-Enkephalin (**12**) in a continuous process by methodA$_{cont}$.

In a round-bottom flask equipped with frit filter, to a solution of an equimolar amount of Z-Phe-OH (**6**) (150 mg, 0.5 mmol, 1.0 eq.) and H-Leu-O^{t}Bu hydrochloride (**7**) (112 mg, 0.5 mmol, 1.0 eq.) in EtOAc (0.1 M), DIPEA (348 µL, 2.0 mmol, 4.0 eq.) and T3P® (50 wt. % in EtOAc, 447 µL, 0.75 mmol, 1.5 eq.) were added following this order. The mixture was stirred at 30° for 5 min, then DIPEA (9 µL, 0.05 mmol, 0.1 eq) and dried Pd/C$_{(10\%)}$ (10% $w_{Pd}/w_{peptide}$, 4.4 mol%) were added and the atmosphere was switched from N_2 to H_2. The reaction mixture was stirred at 30°C for 1 h, then the solution was filtered through the frit filter via nitrogen flow in a double-necked round-bottom flask. The flask was washed with further EtOAc (2 mL) and before iterating the synthetic process with the subsequent coupling, the reaction volume was reduced under nitrogen flow to reach a standard concentration of 47 g/L employed in the next deprotection steps. The same procedure described above was used in subsequent steps for sequence elongation, inserting: Z-Gly-OH (105 mg, 0.5 mmol, 1.0 eq.), Z-Gly-OH (105 mg, 0.5 mmol, 1.0 eq.), and lastly Boc-Tyr(O^{t}Bu)-OH (169 mg, 0.5mmol, 1.0 eq.). A second sequence was synthesized using directly Z-Gly-Gly-OH (133 mg, 0.5 mmol, 1.0 eq.), and then Boc-Tyr(O^{t}Bu)-OH (169 mg, 0.5mmol, 1.0 eq.).

After completion of full sequences, the solution was filtered through a filter paper to remove Pd/C catalyst. The organic layer was washed with 0.1M $HCl_{(aq)}$ (5 mL) and 0.1M $NaHCO_{3(aq)}$ (5 mL), respectively. The organic

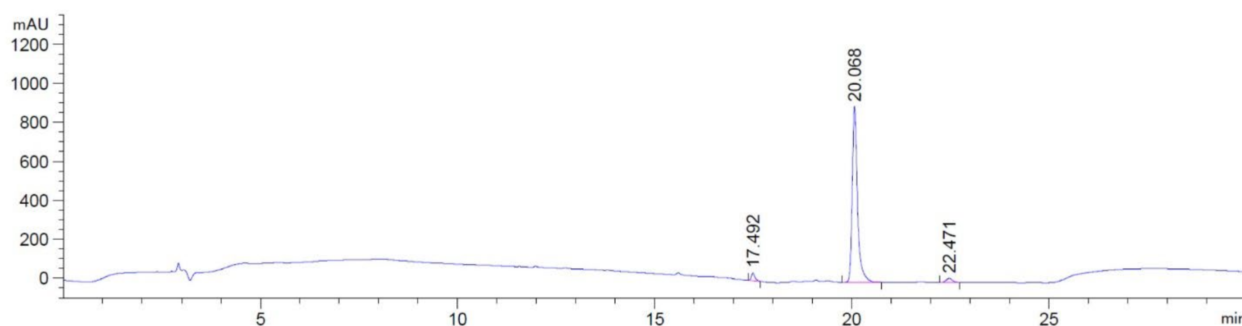
layer was dried over anhydrous Na_2SO_4 and concentrated under vacuum. A white solid was obtained (Yield = 76%). Each step was monitored by HPLC-MS (Analysis Method 3 in Analytical methods section).

HPLC chromatograms



Product	m/z observed	Rt (min)	Area (%)
Boc-Tyr(^{t}Bu)-Gly-Gly-Gly-Phe-Leu-O^{t}Bu	826.4 $[M+H]^+$	18.559	7.9360
Boc-Tyr(^{t}Bu)-Gly-Gly-Phe-Leu-O^{t}Bu (**12**)	768.4 $[M+H]^+$	20.201	92.0640

Figure S45. Chromatogram at 210 nm of fully protected Leu-Enkephalin (**12**) when Z-Gly-OH was employed.



Product	m/z observed	Rt (min)	Area (%)
Boc-Tyr(^{t}Bu)-Gly-Gly-Gly-Gly-Phe-Leu-O^{t}Bu	826.4 $[M+H]^+$	17.492	3.0533
Boc-Tyr(^{t}Bu)-Gly-Gly-Phe-Leu-O^{t}Bu (**12**)	768.4 $[M+H]^+$	20.068	94.2852
Boc-Tyr(^{t}Bu)-Gly-Phe-Leu-O^{t}Bu	711.4 $[M+H]^+$	22.471	2.6616

Figure S46. Chromatogram at 210 nm of fully protected Leu-Enkephalin (**12**) when Z-Gly-Gly-OH was employed.

The synthesis of Leu-Enkephalin using Z-Gly-Gly-OH resulted in 94% purity of the target peptide. However, Glycine (1%) is present in the commercial batch of H-Gly-Gly-OH employed for the synthesis of the starting material Z-Gly-Gly-OH. Therefore, the reported purity is affected also by reagent purity.

9. Synthesis of fully protected Leu-Enkephalin and Pentapeptide-18 (Leuphasyl®) in solution-phase Method A_{cont_plus}

General procedure

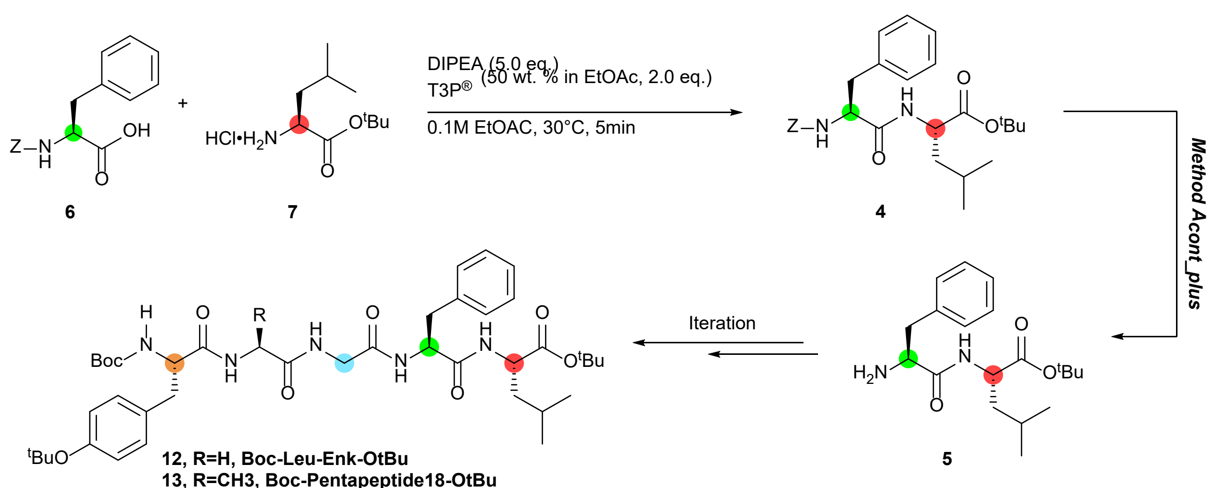
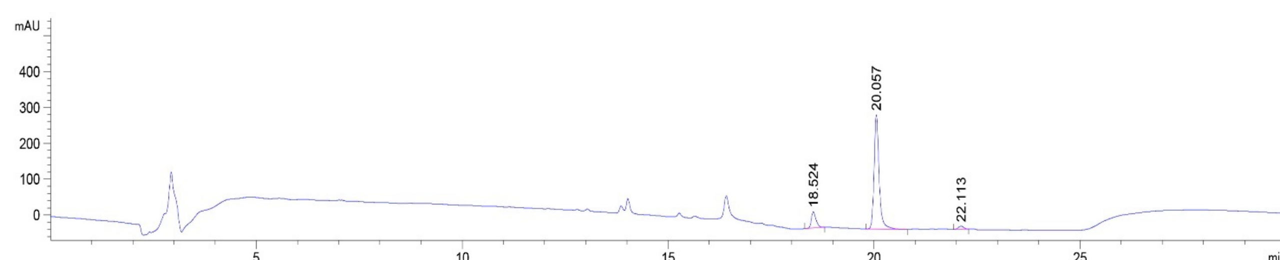


Figure S47. Synthesis of fully protected Leu-Enkephalin (**12**) and Pentapeptide-18 (Leuphasyl®) (**13**) in a continuous process by Method A_{cont_plus}.

An oven-dried, double-neck, round-bottomed flask equipped with stirring bar was charged with Z-Phe-OH (**6**) (150 mg, 0.5 mmol, 1.0 eq.), H-Leu-O^{t}Bu hydrochloride (**7**) (112 mg, 0.5 mmol, 1.0 eq.) in EtOAc (0.1M) under N_2 atmosphere. Subsequently, DIPEA (435 µL, 2.5 mmol, 5.0 eq.) and T3P® (50 wt. % in EtOAc, 596 µL, 1.0 mmol, 2.0 eq.) were added following this order. The solution was stirred for 5 minutes. Then, DIPEA (9 µL, 0.05 mmol, 0.1 eq) and dried Pd/$C_{(10\%)}$ (10% $w_{Pd}/w_{peptide}$, 4.4 mol%) were added and the atmosphere was switched from N_2 to H_2. The mixture was stirred at 30°C for 1h, the nitrogen atmosphere was restored, and the coupling step was carried out without removing Pd/C from the reaction mixture. Following this procedure, the next deprotection steps were carried out using the same catalyst added at the beginning of the iterative process. The same procedure above reported was used in subsequent steps, introducing: Z-Gly-OH (105 mg, 0.5 mmol, 1.0 eq.); Z-Gly-OH (105 mg, 0.5 mmol, 1.0 eq.) or Z-Ala-OH (112 mg, 0.5 mmol, 1.0 eq.); Boc-Tyr(O^{t}Bu)-OH (169 mg, 0.5mmol, 1.0 eq.). After completion of full sequenced elongation, the solution was filtered through a filter paper to remove Pd/C catalyst and the filtrate was washed with 0.1M $HCl_{(aq)}$ (5 mL) and 0.1M $NaHCO_{3(aq)}$ (5 mL). The organic layer was dried over anhydrous Na_2SO_4 and concentrated under vacuum. A white solid was obtained (Yield = 78% for **12** and Yield = 82% for **13**). Each step was monitored by HPLC-MS analysis (Analysis Method 3 in Analytical methods section).

HPLC chromatogram



Product	m/z observed	Rt (min)	Area (%)
Boc-Tyr(^{t}Bu)-Gly-Gly-Gly-Phe-Leu-O^{t}Bu	825.4 $[M+H]^+$	18.524	11.3258
Boc-Tyr(^{t}Bu)-Gly-Gly-Phe-Leu-O^{t}Bu (**12**)	768.4 $[M+H]^+$	20.057	86.0781
Boc-Tyr(^{t}Bu)-Gly-Phe-Leu-O^{t}Bu	711.5 $[M+H]^+$	22.113	2.5961

Figure S48. Chromatogram at 210 nm of fully protected Leu-Enkephalin (**12**). The peak at 16.3 min is related to Toluene.

10. Synthesis of fully protected Leu-Enkephalin in solution-phase by Method B_{cont}

General procedure

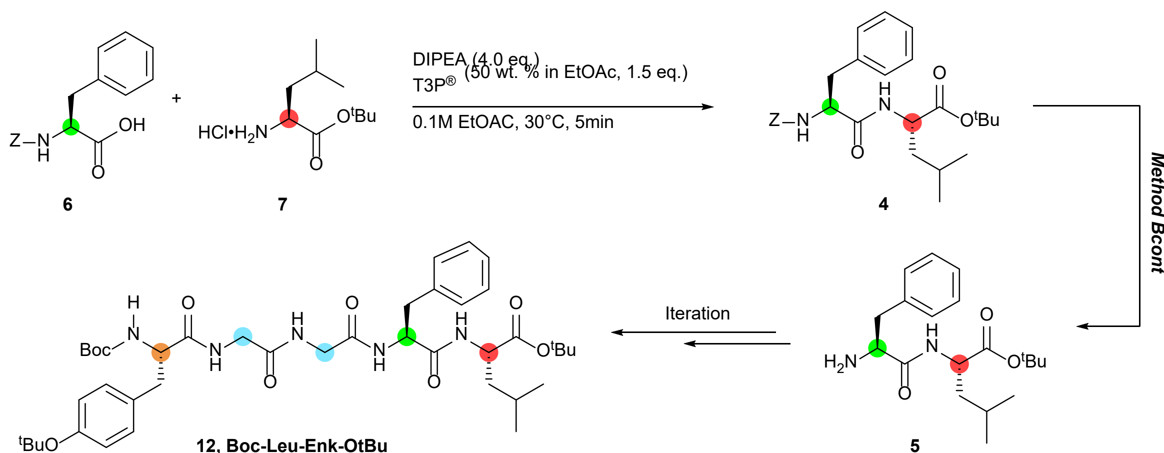
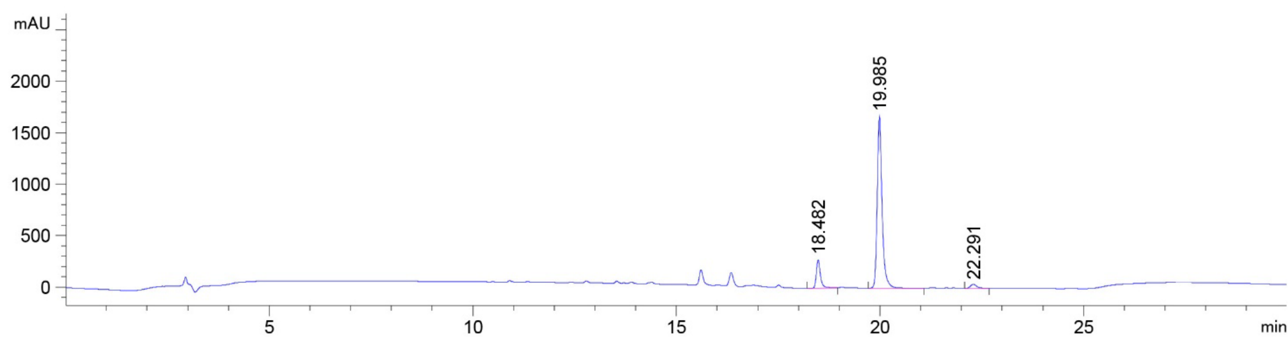


Figure S49. Synthesis of fully protected Leu-enkephalin (**12**) in a continuous process by method B_{cont}.

An oven-dried, double-neck, round-bottomed flask equipped with stirring bar was charged with Z-Phe-OH (**6**) (150 mg, 0.5 mmol, 1.0 eq), H-Leu-O^{t}Bu hydrochloride (**7**) (112 mg, 0.5 mmol, 1.0 eq.) in EtOAc (0.1M) under N_2 atmosphere. Subsequently, DIPEA (348 µL, 2.0 mmol, 4.0 eq.) and T3P® (50 wt. % in EtOAc, 447 µL, 0.75 mmol, 1.5 eq.) were added following this order. The solution was stirred for 5 minutes and then it was directly

injected in H-CUBE® following the procedure reported in Method B (Par 6.c, Supp. Info page S28). The reaction mixture was collected in an oven-dried flask purged under N_2 atmosphere and the excess of solvent was evaporated by reduce pressure preserving the system under N_2 atmosphere to reach a standard concentration of 47 g/L employed in the next deprotection steps. The same procedure above mentioned was used in subsequent steps, introducing: Z-Gly-OH (105 mg, 0.5 mmol, 1.0 eq.); Z-Gly-OH (105 mg, 0.5 mmol, 1.0 eq.); Boc-Tyr(O^{t}Bu)-OH (169 mg, 0.5mmol, 1.0 eq.). After completion of full sequenced elongation, the solution was filtered through a filter paper to remove Pd/C catalyst and the filtrate was washed with 0.1M $HCl_{(aq)}$ (5 mL) and 0.1M $NaHCO_{3(aq)}$ (5 mL). The organic layer was dried over anhydrous Na_2SO_4 and concentrated under vacuum. A white solid was obtained (Yield = 81%). Each step was monitored by HPLC-MS analysis (Analysis method 3 in Analytical methods section).

HPLC chromatogram



Product	m/z observed	Rt (min)	Area (%)
Boc-Tyr(^{t}Bu)-Gly-Gly-Gly-Phe-Leu-O^{t}Bu	825.4 $[M+H]^+$	18.482	11.6198
Boc-Tyr(^{t}Bu)-Gly-Gly-Phe-Leu-O^{t}Bu (**12**)	768.4 $[M+H]^+$	19.985	85.9404
Boc-Tyr(^{t}Bu)-Gly-Phe-Leu-O^{t}Bu	711.4 $[M+H]^+$	22.291	2.4399

Figure S50. Chromatogram at 210 nm of fully protected Leu-Enkephalin (**12**). The peak at 16.3 min is related to Toluene.

11. Complete deprotection of fully protected peptides

General Procedure

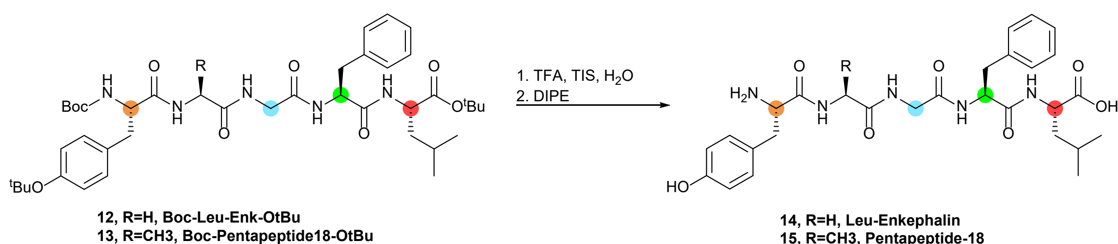
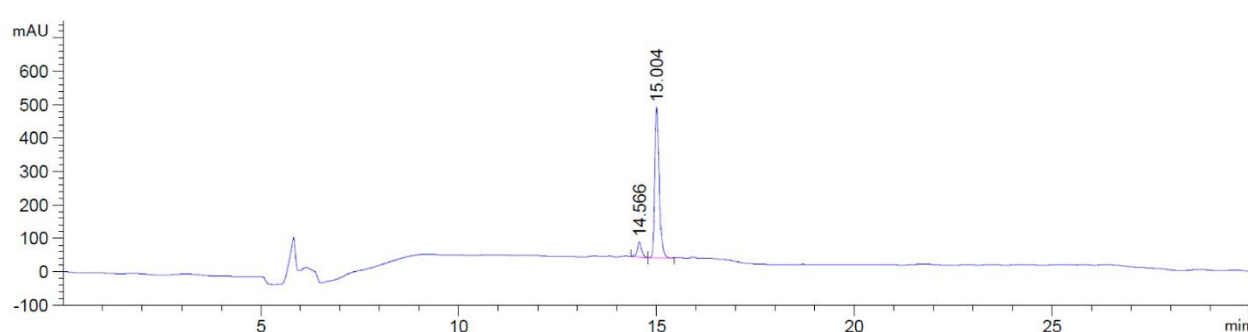


Figure S51. Acidic removal of protecting groups on fully protected Leu-Enkephalin and Pentapetide-18 (Leuphasyl).

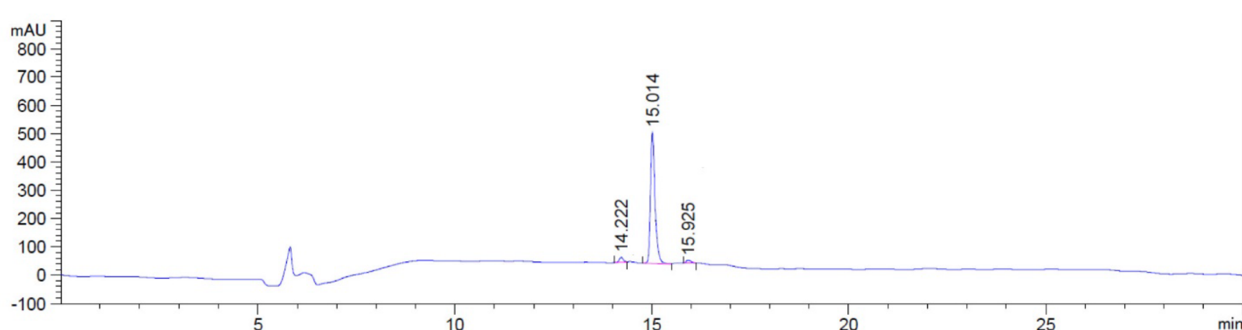
In a round-bottom flask, the fully protected peptide (**12** or **13**) was dissolved in a deprotection cocktail (0.08 M) composed by: 95.0% TFA, 2.5% H_2O, and 2.5% TIS. The reaction mixture was stirred at room temperature for 2 h, then 15 mL of DIPE were added dropwise inducing the formation of a white solid. After the complete addition of the solvent, the reaction mixture was cooled to 0°C and slowly stirred for 30 min. Subsequently, the white solid was recovered by filtration. The purity of the target peptide was monitored by HPLC-MS analysis (Analysis Method 4 in Analytical methods section).

HPLC chromatogram



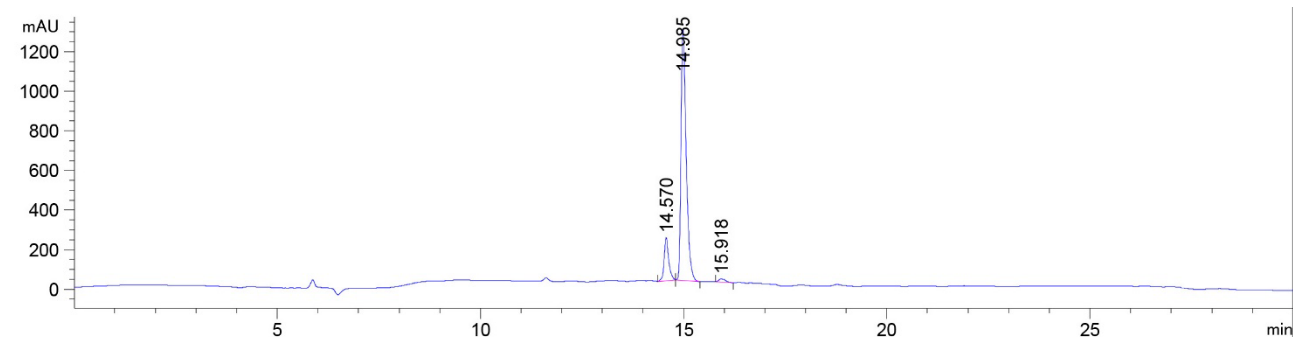
Product	m/z observed	Rt (min)	Area (%)
H-Tyr-Gly-Gly-Gly-Phe-Leu-OH	613.2 $[M+H]^+$	14.566	9.1682
H-Tyr-Gly-Gly-Phe-Leu-OH (**14**)	556.2 $[M+H]^+$	15.004	90.8318

Figure S52. Chromatogram at 210 nm of deprotected Leu-Enkephalin (**14**) by method A_{cont}.



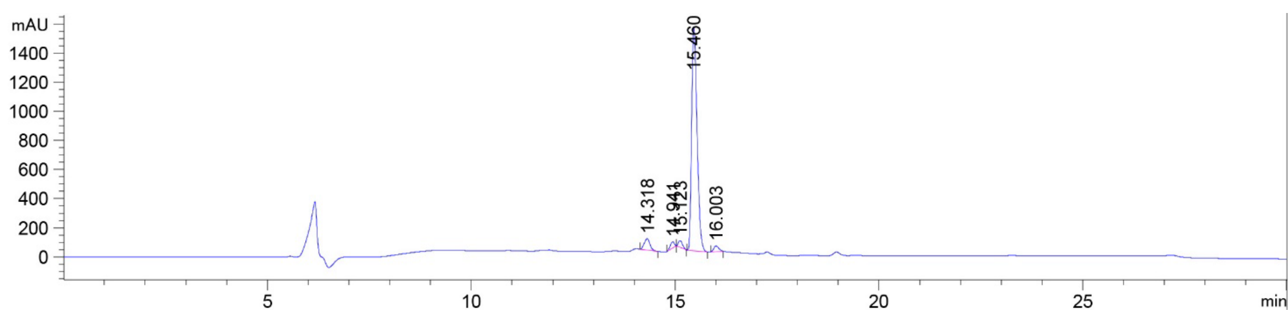
Product	m/z observed	Rt (min)	Area (%)
H-Tyr-**Gly**-Gly-Gly-Phe-Leu-OH	613.2 $[M+H]^+$	14.222	3.1811
H-Tyr-Gly-Gly-Phe-Leu-OH (**14**)	556.2 $[M+H]^+$	15.014	94.4799
H-Tyr-Gly-Phe-Leu-OH	499.1 $[M+H]^+$	15.925	2.3380

Figure S53. Chromatogram at 210 nm of deprotected Leu-Enkephalin (**14**) employing Z-Gly-Gly-OH by method A_{cont}.



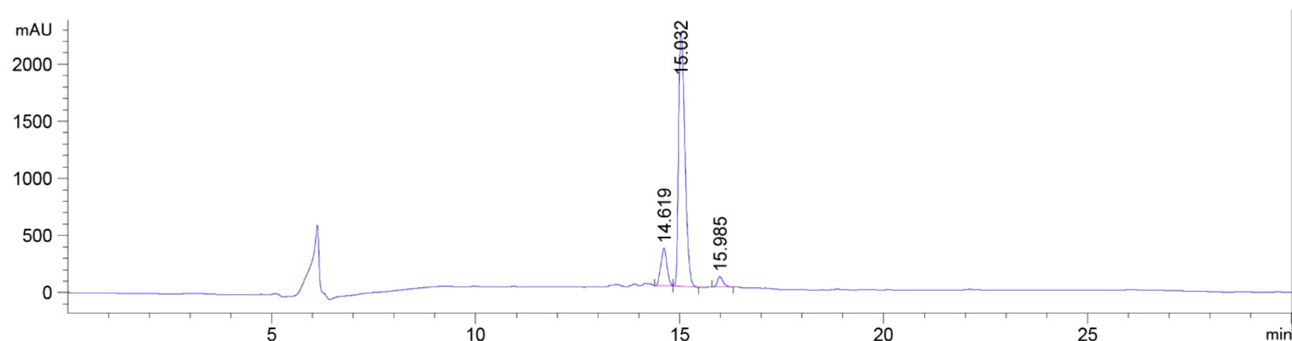
Product	m/z observed	Rt (min)	Area (%)
H-Tyr-Gly-Gly-Gly-Phe-Leu-OH	613.2 $[M+H]^+$	14.570	13.0769
H-Tyr-Gly-Gly-Phe-Leu-OH (**15**)	556.2 $[M+H]^+$	14.985	85.6142
H-Tyr-Gly-Phe-Leu-OH	499.1 $[M+H]^+$	15.918	1.3089

Figure S54. Chromatogram at 210 nm of deprotected Leu-Enkephalin (**14**) by method A_{cont_plus}.



Product	m/z observed	Rt (min)	Area (%)
H-Ala-Gly-Phe-Leu	407.1$[M+H]^+$	14.318	5.0535
H-Tyr-Ala-Gly-Gly-Phe-Leu-OH	627.3$[M+H]^+$	14.941	1.7817
H-Tyr-Ala-Ala-Gly-Phe-Leu-OH	641.3 $[M+H]^+$	15.123	2.0552
H-Tyr-Ala-Gly-Phe-Leu-OH (**15**)	570.3 $[M+H]^+$	15.460	88.9988
H-Tyr-Ala-Phe-Leu-OH	513.2 $[M+H]^+$	16.003	2.1108

Figure S55. Chromatogram at 210 nm of Pentapeptide-18 (**15**) by method A_{cont_plus}.



Product	m/z observed	Rt (min)	Area (%)
H-Tyr-Gly-Gly-Gly-Phe-Leu-OH	613.2 $[M+H]^+$	14.619	11.7423
H-Tyr-Gly-Gly-Phe-Leu-OH (**14**)	556.2 $[M+H]^+$	15.032	85.2429
H-Tyr-Gly-Phe-Leu-OH	499.2 $[M+H]^+$	15.985	3.0148

Figure S56. Chromatogram at 210 nm of deprotected Leu-Enkephalin (**14**) by method B_{cont}.

12. PMI calculation for the synthesis of Leu-Enkephalin

PMI is defined as the ratio between the total mass of materials and the mass of the isolated product, as reported below:

$$PMI = \frac{\sum mass\ of\ materials}{mass\ of\ the\ isolated\ product}$$

In this case, the total mass of materials includes:

- Reagents: Z-AA-OH and HCl, H_2N-Leu-O^{t}Bu, DIPEA, T3P® (50 wt. % in EtOAc), TFA, scavengers (TIPS and H_2O);
- Solvents: EtOAc (reaction medium and washing/extraction steps), ^{i}PrOH (H-CUBE® washing), HCl 0.1M and $NaHCO_3$ (washing steps), DIPE (crude peptide precipitation);
- Catalyst (Pd/$C_{(10\%)}$) and H_2.
- Other: Na_2SO_4

When solvents (EtOAc and ^{i}PrOH) and base (DIPEA) were recovered, the value of the final PMI was calculated by subtracting the mass of recovered materials from the mass of used materials, according to the following equation:

$$PMIr = \frac{\sum mass\ of\ materials - \sum mass\ of\ recovered\ materials}{mass\ of\ the\ isolated\ product}$$

DIPEA was recovered by extraction (80% $_{w/w}$); EtOAc, water and ^{i}PrOH were recovered by distillation (90% $_{w/w}$).

Furthermore, Pd/C in the cartridge (H-CUBE®) has a less relevant effect on the PMI since the catalyst can be used many times and, in our experience, the efficiency of the catalyst remained unchanged after 20 cycles. This result is in agreement with the literature.[3] For the procedures in bench, Pd/C was filtered and collected to be regenerated later. The recovery of Pd/C was not included in the calculations.

12.a Calculation of total mass of reagents employed for the synthesis of Leu-enkephalin using Method A_{cont}, Method A_{cont_plus} and Method B

Table S9. Calculation of Process Mass Intensity (PMI) and Process Mass Intensity after recovery (PMIr) for the synthesis of Leu-Enkephalin with Method $A_{cont_}$

Component	Contribution to PMI (g/g product)	Contribution to PMIr (g/g product)
Amino acids	2.52	2.52
T3P®	6.11	6.11
DIPEA	5.26	1.05
EtOAc	128.37	12.84
Pd/$C_{(10\%)}$	0.31	0.31
H_2	0.04	0.04
H_2O	79.23	79.23
Cleavage and washing components (HCl, NaOH, $NaHCO_3$, Na_2SO_4 TFA, TIS)	34.77	34.77
DIPE	42.81	42.81
Total	**299.43**	**179.68**

Table S10. Calculation of Process Mass Intensity (PMI) and Process Mass Intensity after recovery (PMIr) for the synthesis of Leu-Enkephalin with Method $B_{cont_}$ The amount of hydrogen was calculated based on water consumed by the instrument generating H2.

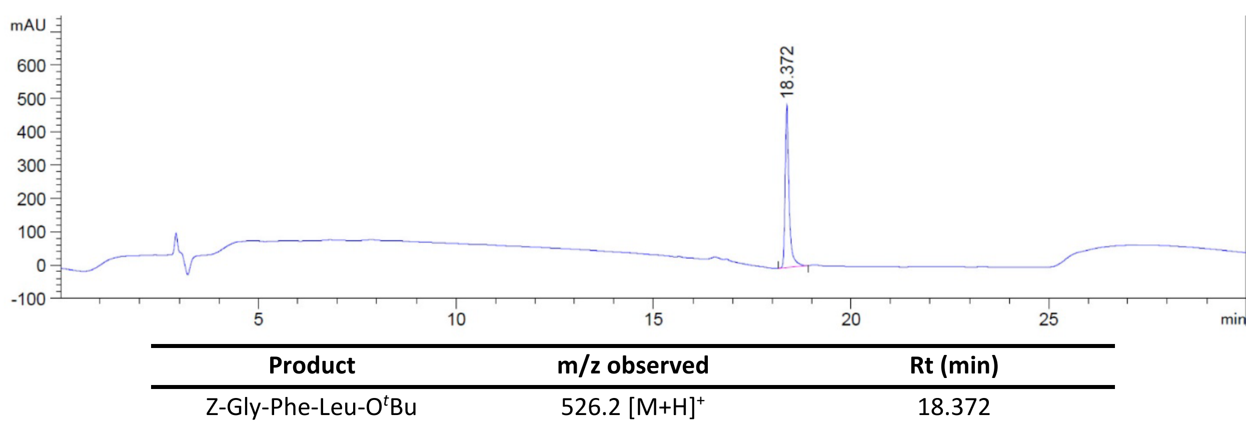
Component	Contribution to PMI (g/g product)	Contribution to PMIr (g/g product)
Amino acids	2.37	2.37
T3P®	4.86	4.86
DIPEA	4.21	0.84
EtOAc	300.28	30.03
Pd/$C_{(10\%)}$	0.02	0.02
H_2O	80.09	80.09
^{i}PrOH	87.33	8.73
Cleavage and washing components (HCl, NaOH, $NaHCO_3$, Na_2SO_4 TFA, TIS)	32.71	32.71
DIPE	40.28	40.28
Total	**552.15**	**199.93**

Table S11. Calculation of Process Mass Intensity (PMI) and Process Mass Intensity after recovery (PMIr) for the synthesis of Leu-Enkephalin with Method A_{cont_plus}

Component	Contribution to PMI (g/g product)	Contribution to PMIr (g/g product)
Amino acids	2.46	2.46
T3P®	5.51	5.51
DIPEA	4.72	0.94
EtOAc	71.6	7.16
Pd/C(10%)	0.09	0.09
H_2	0.02	0.02
H_2O	38.94	38.94
Cleavage and washing components (HCl, NaOH, $NaHCO_3$, Na_2SO_4 TFA, TIS)	31.65	31.65
DIPE	41.83	41.83
Total	**196.85**	**128.60**

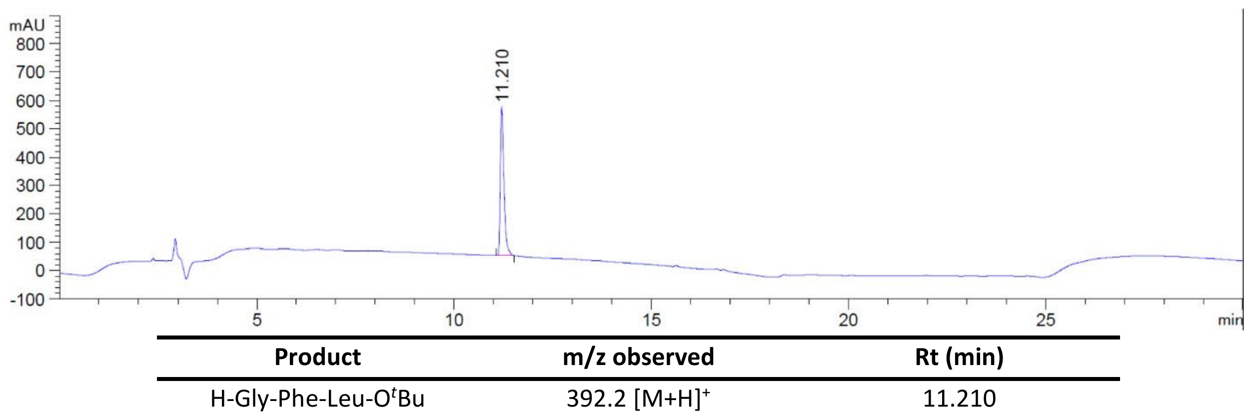
13. Annex

In this section, chromatograms of all the species observed during the synthesis of Leu-Enkephalin are reported. In addition, the two main impurities (double hit of Glycine and des-Gly) are reported as both fully protected and deprotected peptides. All the species were synthesized in a step-by-step process, performing the proper work-up after each reaction to remove any trace of starting materials or solvents. Z-Phe-Leu-O^{t}Bu and H-Phe-Leu-O^{t}Bu chromatograms were already reported in the Par. 5 and 6 (Supp. Info pages S16 and S28). Moreover, in the caption of each chromatogram is specified the HPLC-MS analysis employed.



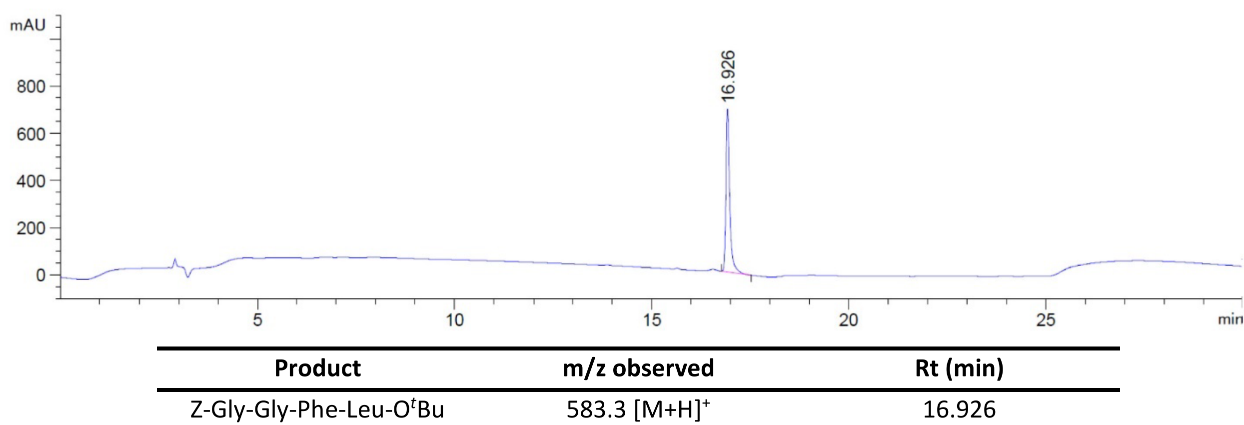
Product	m/z observed	Rt (min)
Z-Gly-Phe-Leu-O^{t}Bu	526.2 $[M+H]^+$	18.372

Figure S57. Chromatogram at 210 nm of fully protected Z-Gly-Phe-Leu-O^{t}Bu. Analysis method 3 in Analytical Methods.



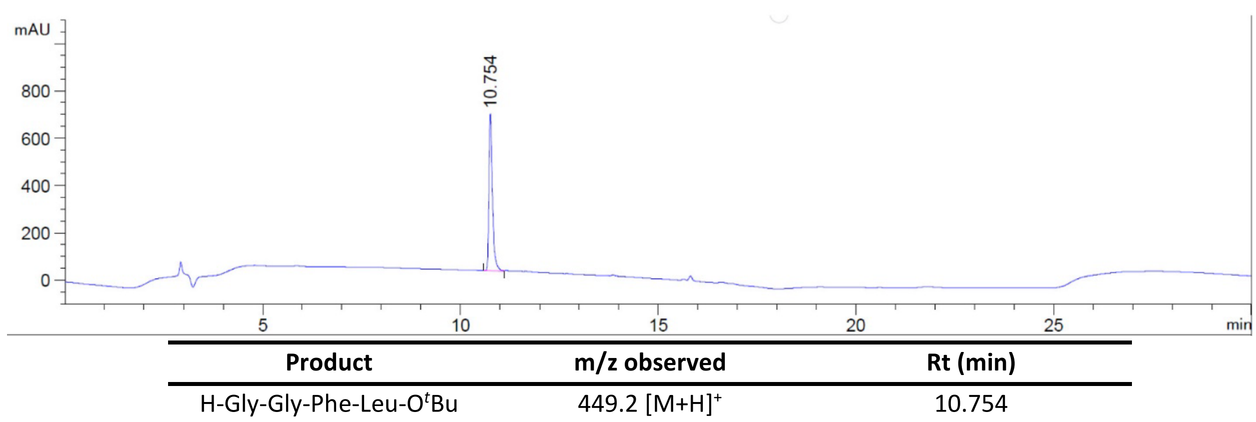
Product	m/z observed	Rt (min)
H-Gly-Phe-Leu-O^{t}Bu	392.2 [M+H]$^+$	11.210

Figure S58. Chromatogram at 210 nm of fully protected H-Gly-Phe-Leu-O^{t}Bu. Analysis method 3 in Analytical Methods.



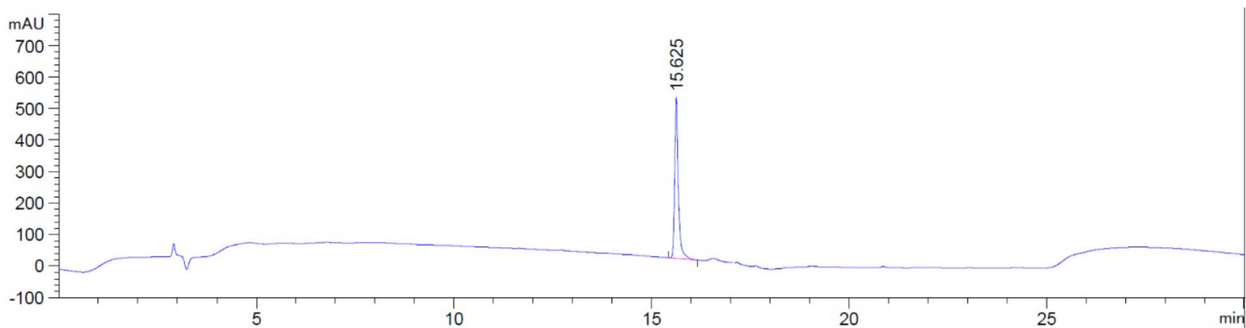
Product	m/z observed	Rt (min)
Z-Gly-Gly-Phe-Leu-O^{t}Bu	583.3 [M+H]$^+$	16.926

Figure S59. Chromatogram at 210 nm of fully protected Z-Gly-Gly-Phe-Leu-O^{t}Bu. Analysis method 3 in Analytical Methods.



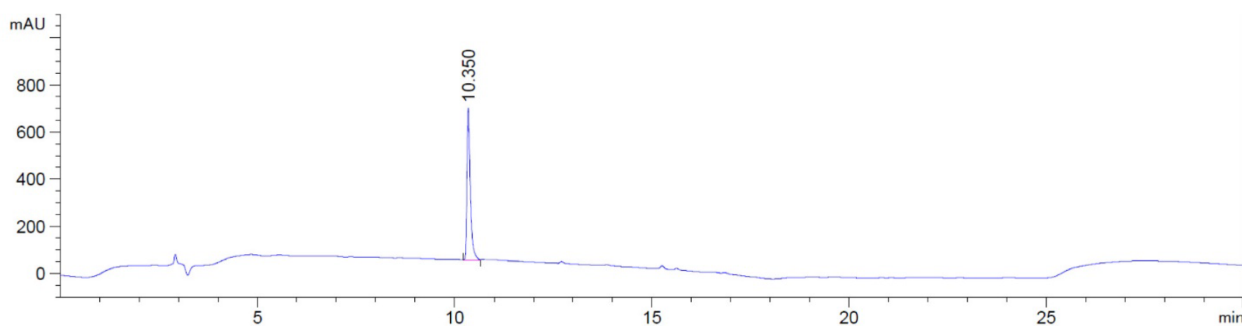
Product	m/z observed	Rt (min)
H-Gly-Gly-Phe-Leu-O^{t}Bu	449.2 [M+H]$^+$	10.754

Figure S60. Chromatogram at 210 nm of fully protected H-Gly-Gly-Phe-Leu-O^{t}Bu. Analysis Method 3 in General Methods.



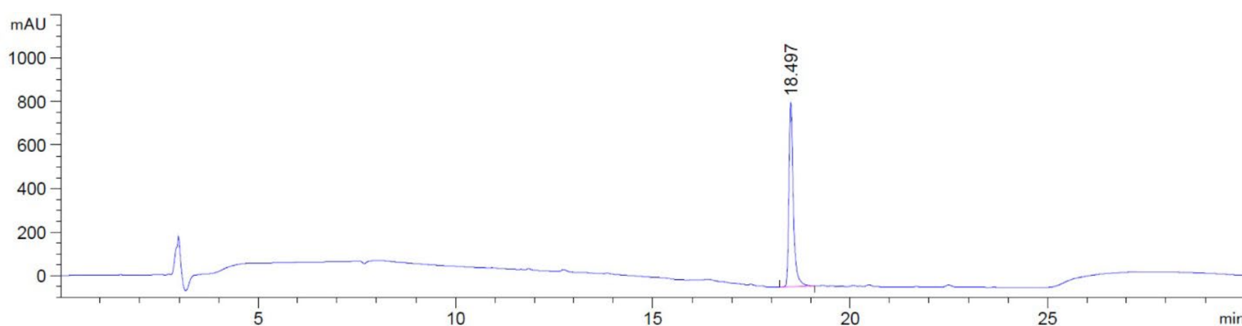
Product	m/z observed	Rt (min)
Z-Gly-Gly-Gly-Phe-Leu-O^{t}Bu	640.3 $[M+H]^+$	15.625

Figure S61. Chromatogram at 210 nm of fully protected Z-Gly-Gly-Gly-Phe-Leu-O^{t}Bu. Analysis method 3 in Analytical Methods.



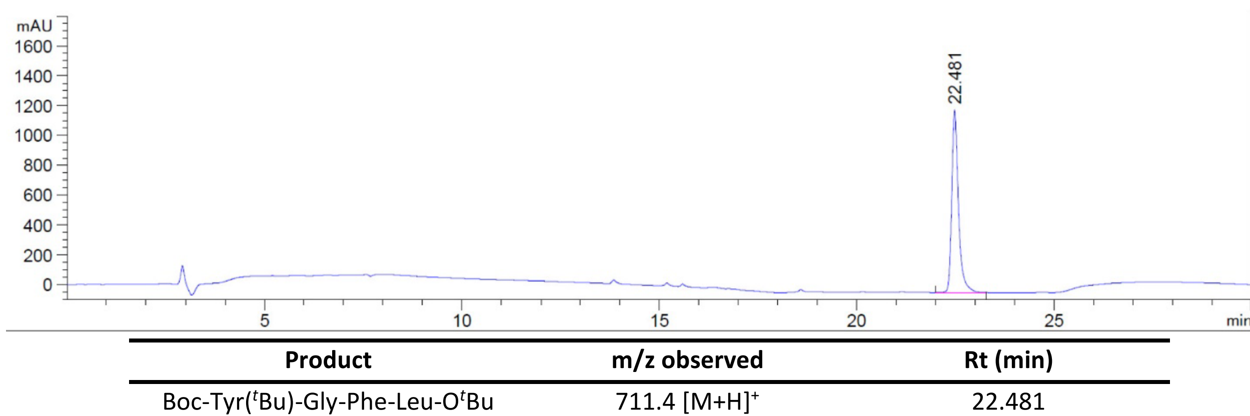
Product	m/z observed	Rt (min)
H-Gly-Gly-Gly-Phe-Leu-O^{t}Bu	506.2 $[M+H]^+$	10.350

Figure S62. Chromatogram at 210 nm of fully protected H-Gly-Gly-Gly-Phe-Leu-O^{t}Bu. Analysis method 3 in Analytical Methods.



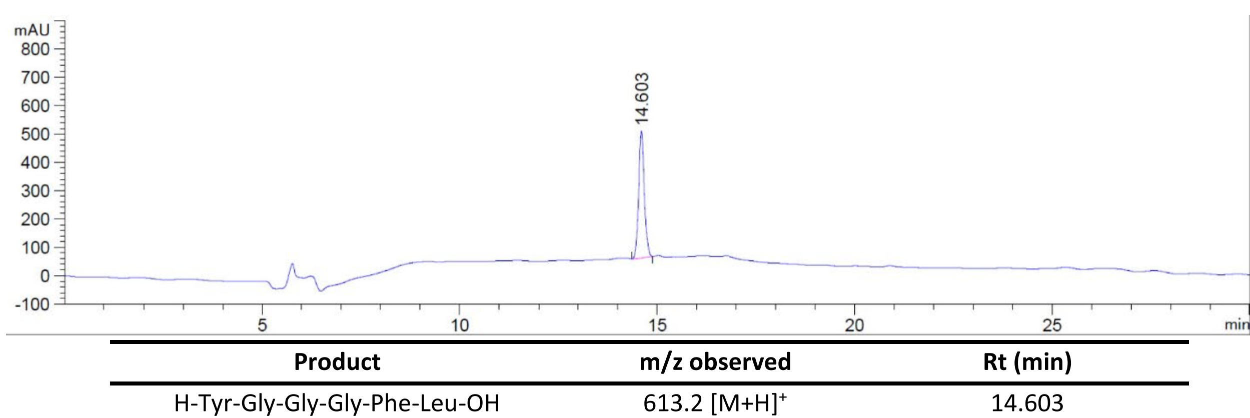
Product	m/z observed	Rt (min)
Boc-Tyr(^{t}Bu)-Gly-Gly-Gly-Phe-Leu-O^{t}Bu	825.5 $[M+H]^+$	18.497

Figure S63. Chromatogram at 210 nm of fully protected Boc-Tyr(^{t}Bu)-Gly-Gly-Gly-Phe-Leu-O^{t}Bu. Analysis method 3 in Analytical Methods.



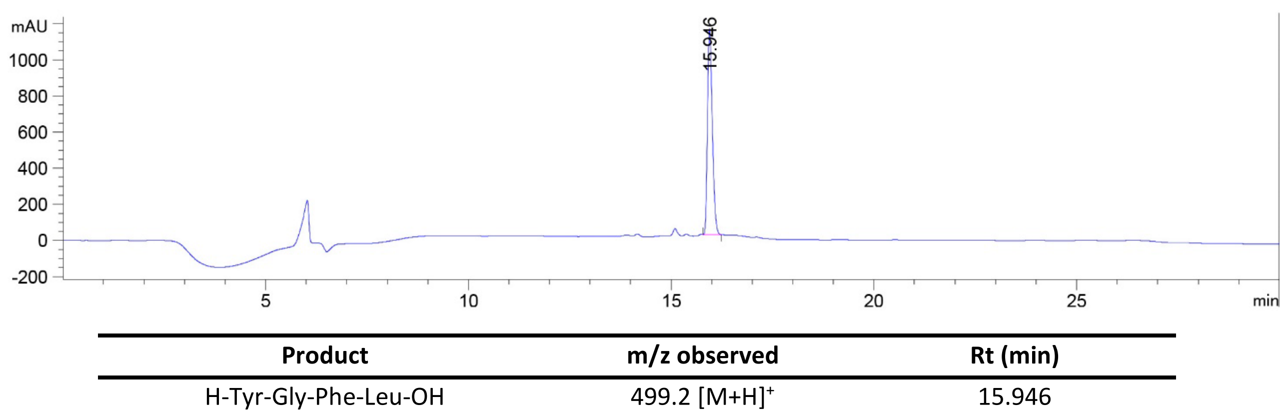
Product	m/z observed	Rt (min)
Boc-Tyr(^{t}Bu)-Gly-Phe-Leu-O^{t}Bu	711.4 [M+H]$^+$	22.481

Figure S64. Chromatogram at 210 nm of fully protected Boc-Tyr(^{t}Bu)-Gly-Phe-Leu-O^{t}Bu. Analysis method 3 in Analytical Methods.



Product	m/z observed	Rt (min)
H-Tyr-Gly-Gly-Gly-Phe-Leu-OH	613.2 [M+H]$^+$	14.603

Figure S65. Chromatogram at 210 nm of fully protected H-Tyr-Gly-Gly-Gly-Phe-Leu-OH. Analysis method 4 in Analytical Methods.



Product	m/z observed	Rt (min)
H-Tyr-Gly-Phe-Leu-OH	499.2 [M+H]$^+$	15.946

Figure S66. Chromatogram at 210 nm of fully protected H-Tyr-Gly-Phe-Leu-OH. Analysis method 4 in Analytical Methods.

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