Electronic Supplementary Material (ESI) for Chemical Science.

Supporting Information

A versatile *o*-aminoanilide linker for native chemical ligation

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1. Materials and methods

1.1. Chemicals

Reagent	Vendor
Silver triflate (AgOTf), acetic acid, *N,N*-diisopropylethylamine (DIEA), *N,N'*-diisopropylcarbodiimide (DIC), K_2CO_3, *o*-phenylenediamine, zinc chloride ($ZnCl_2$), sodium hydrosulfite (NaS_2O_4), triisopropylsilane (TIS), pyridine, *p*-nitrophenyl chloroformate, Tergitol® solution (type NP-40, 70% in H_2O), 2,4,6-trimethylpyridine	Sigma-Aldrich
DCM, Et_2O, DMF (peptide synthesis grade), piperidine, ACN (HPLC grade)	Carlo Erba
Triphosgene, sodium 2-mercaptoethanesulfonate (MESNa), trifluoroacetic acid (TFA, peptide grade), 2-fluoronitrobenzene, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), 2-(1H-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU), tris(2-carboxyethyl)phosphine (TCEP·HCl)	Fluorochem
Phenyl chloroformate, diphenyl diselenide (DPDS), DL-dithiothreitol (DTT)	TCI Chemicals
Fmoc and Boc-amino acids	Iris Biotech
4-mercaptophenol (4-MPOH)	Apollo Scientific
Tetrabutylammonium hydrogen sulfate (TBAHS)	Fluka
1-hydroxy-6-chloro-benzotriazole (6-Cl-HOBt)	Luxembourg Ind.
p-cyanophenol, guanidine hydrochloride (Gdm.HCl, 99.5%, without anticaking agent)	Acros Organics
Sodium nitrite ($NaNO_2$)	Panreac
Resins	
Aminomethylated polystyrene LL (0.36 mmol/g), Aminomethyl NovaGel (0.69 mmol/g), NovaSyn TG amino (0.29 mmol/g), Aminomethylated polystyrene LL (0.46 mmol/g)	Novabiochem
Aminomethyl-ChemMatrix (0.56 mmol/g), 2-chlorotrityl chloride (1.6 mmol/g), Fmoc-Rink Amide AM (0.74 mmol/g)	Iris Biotech
PL-PEGA (0.2 mmol/g)	Polymer Laboratories
TentaGel S AM (0.4 mmol/g)	Advanced ChemTech
Aminomethylated polystyrene resin.HCl (0.62 mmol/g)	Peptides International
Fmoc-PAL-PEG-PS (0.18 mmol/g)	Applied Biosystems
TentaGel HL NH_2 (0.54 mmol/g)	Rapp Polymere GmbH

p-cyanophenyl chloroformate was synthesized following reported procedures.[1] Fmoc-PAL (Fmoc-4-(aminomethyl)-3,5-dimethoxyphenoxy)butanoic acid) was kindly provided by Dr. Fernando Albericio.[2] Commercially available versions of this linker can be purchased from Iris Biotech, Advanced ChemTech, Sigma-Aldrich.

1.2. Instrumentation

Analytical RP-HPLC was performed on a Hewlett Packard 1100 equipped with an automatic injector and hyphenated to a G1315A photodiode array. Separations were carried out in the following columns:

- Column A: Phenomenex Aeris Peptide 3.6 µm XB-C_{18}, 150 x 4.60 mm
- Column B: Atlantis T3 3 µm C_{18}, 100 x 4.60 mm
- Column C: Vydac Protein & Peptide C_{18}, 250 x 4.60 mm

Runs were carried out at room temperature using a linear gradient of 0 to 46% of buffer B (ACN/TFA, 0.036% TFA) in buffer A (H_2O/TFA, 0.045% TFA) during 20 min (unless otherwise stated) at a flow rate of 1 mL/min. UV detection was done at 220, 247 and 280 nm. Chromatograms were analyzed using the HP Chemstation software and processed with the MestreNova MS Plugin. All the HPLC chromatograms showed in the manuscript and supporting information correspond to the 220 nm trace.

Semi-preparative HPLC was performed on a Waters PrepLC system furnished with a Waters 1525 Binary HPLC Pump and a Waters 2489 UV/Visible Detector. Separation was undertaken in a XBridge Prep BEH130 C_{18} 5 µm OBD 19 x 50 mm column. Runs were performed using a linear gradient of H_2O/TFA 0.1% (buffer A) and ACN/TFA 0.05% (buffer B), from 5% to 70% of B over 85 min at a flow rate of 16 mL/min. UV monitoring was done at 220 nm and 280 nm.

MALDI-TOF mass spectrometry was carried out on a Bruker Daltonics Autoflex III Smartbeam. Spectra were obtained in reflector (<2500 Da) or in linear mode (>2500 Da), analyzed using the Bruker Flexanalysis 3.4 software and processed using the MestreNova MS Plugin.

LC-ESIMS was performed in a Waters LC-MS system consisting in a Waters 2795 separation module hyphenated to a Waters 2996 photodiode array detector and a Waters Micromass ZQ. Separations were carried out in a Waters XSelect C_{18} column (3.5 µm, 50 x 6.0 mm) at 50 °C using a linear gradient of H_2O/formic acid 0.1% (solvent A) and ACN/formic acid 0.07% (solvent B), from 5% to 100% of B over 5 min. UV monitoring was done by diode array detection. Bayesian deconvolution of mass spectrum was performed using the UniDec software developed by Marty et al.[3]

Circular dichroism experiments were performed in a J-715 spectropolarimeter (Jasco Inc.) and registered using the Spectra Manager version 1.53.01. The following parameters were used: range = 300 - 190 nm, data pitch = 0.5 nm, scanning speed= 100 nm/min, response = 1 s, band width = 1.0 nm, accumulation = 5, integration time = 4 s. Spectra were slightly smoothened using the 'smooth' macros of KaleidaGraph.

Water was purified using a Millipore purification system.

2. Solid phase peptide synthesis of the Dbz-PAL-resin and peptideXaa_1-Dbz-PAL-resin

The indicated aminomethylated resin (1 g) was swollen, first in DCM and then in DMF, in a polypropylene syringe. Next, Fmoc-PAL (3 eq) preactivated with HATU (3 eq) and DIEA (4.5 eq) in a DMF solution (30 s), was added and the resulting mixture gently stirred for 1 h. Following the removal of the Fmoc group (actual loading of the resin calculated based on the Fmoc-dibenzofulvene adduct), 2-fluoronitrobenzene (1 mL, 9.5 mmol) and DIEA (0.5 mL, 2.9 mmol) were added in DMF and the solution stirred overnight at room temperature.

NO_2 reduction of PEG-based resins: the (2-PAL)nitrobenzene-resin was first swollen in H_2O, and then transferred to a conical polypropylene tube (50 mL falcon tube). An aqueous solution of $Na_2S_2O_4/K_2CO_3$ (1:1, 0.5 M, 40 mL) was added and the mixture gently shaken (10 rpm) in an orbital shaker for 12 h. Next, the resin was transferred back to the polypropylene syringe, rinsed with H_2O and DMF.

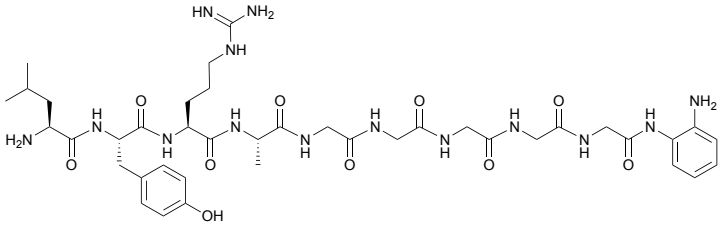
NO_2 reduction of polysteryne resins: the (2-PAL)nitrobenzene-resin was transferred to a conical polypropylene tube (50 mL falcon tube). DCM (20 mL) was added and the resin let to swell for 30 min. Then, an aqueous solution of $Na_2S_2O_4/K_2CO_3$ (1:1, 1.0 M, 20 mL) containing TBAHS (0.8 g) was added and the mixture gently shaken (10 rpm) in an orbital shaker for 12 h. The next day, the resin was filtered off and washed with H_2O and DMF.

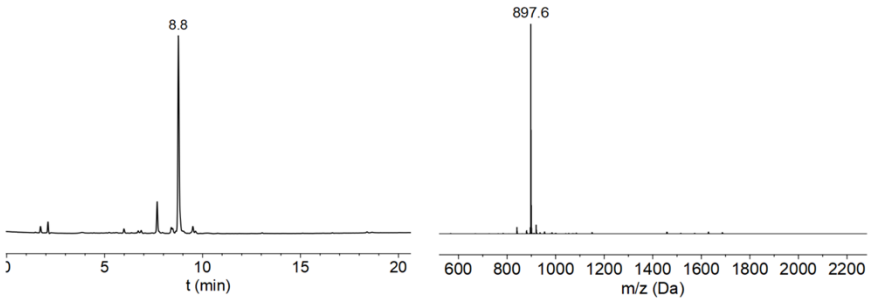
Coupling of the first Fmoc-aa (Xaa_1) was accomplished using the following conditions: Fmoc-aa (5 eq), preactivated with HBTU/HATU (5 eq) and DIEA (7.5 eq) in DMF for 30 s, 1 h coupling (HBTU for Fmoc-Gly and HATU for all other Fmoc-aa). The efficiency of the NO_2 reduction was calculated following the removal of the Fmoc group (Fmoc-dibenzofulvene) after the incorporation of this first amino acid.

The elongation of the peptide sequence was performed under standard Fmoc-SPPS protocols: Fmoc-aa (5 eq), preactivated with HBTU (5 eq) and DIEA (7.5 eq) in DMF for 30 s, 1 h coupling. Fmoc removal was carried out by piperidine (20% in DMF) treatments (2x5 min). Cleavage of the peptide was performed using the cocktail TFA/TIS/H_2O (95:2.5:2.5) for 1 h 30 min (peptideXaa_1-Dbz) or 2 h 30 min (peptideXaa_1-(*p*CN-Phoc)Dbz). The TFA solution containing the peptide was evaporated to almost dryness, and the peptide precipitated over cold Et_2O. The mixture was spun out and the collected pellet separated from the Et_2O by decantation. The solid was dissolved in a H_2O/ACN (1:1) mixture and lyophilized.

2.1. Synthesis of $LYRAG_5$-Dbz

The synthesis was carried out on an aminomethylated polysterene LL (0.46 mmol/g, 0.5 g, Novabiochem). Following the incorporation of Dbz-PAL, the Fmoc-amino acids (1.0 mmol) were incorporated following standard protocols of Fmoc-SPPS: preactivation with HBTU (1.0 mmol) and DIEA (1.5 mmol) in a DMF solution (2 mL, 30 s) and stirring for 1 h. Fmoc removal was carried out with a solution of piperidine (20%) in DMF (2x5 min). Leu was introduced as Boc-Leu.



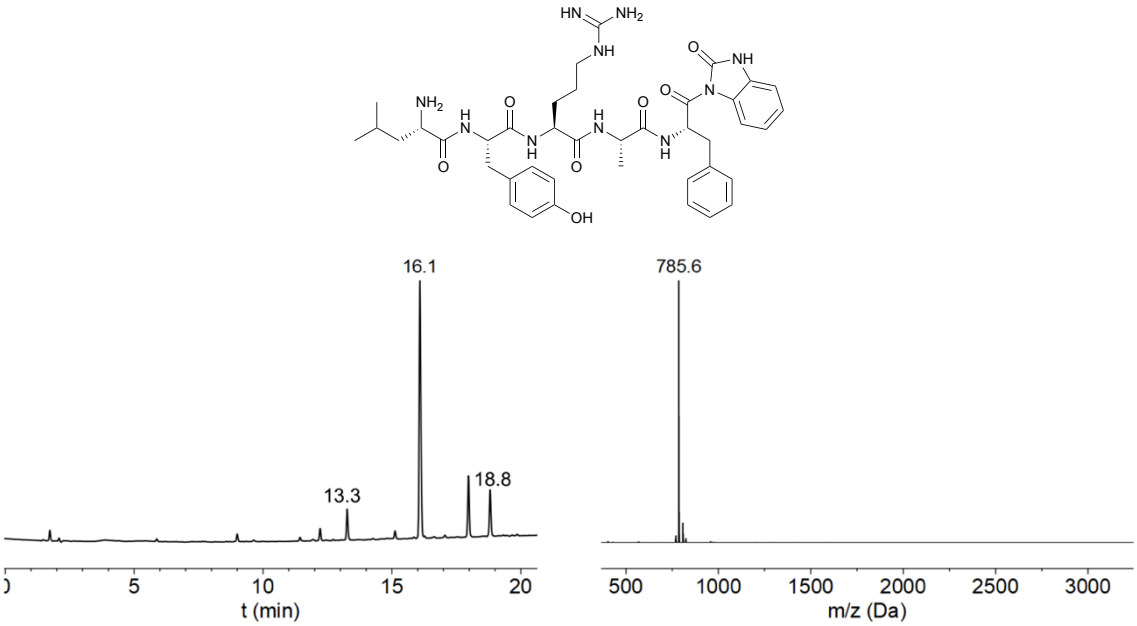


Column A. Calc. $[M+H]^+$: 898.0, found: 897.6.

Figure S1. LYRAG$_5$-Dbz. HPLC trace of the crude peptide synthesis and MALDI-TOF (right). Compound corresponding to the peak at 8.7 min has the expected MW (897.6).

2.2. Synthesis of LYRAF-Nbz

The synthesis was carried out in an aminomethylated polystyrene LL (0.36 mmol/g, Novabiochem) on a 0.5 g scale. Following chain assembly, 200 mg of peptidyl-resin were carbamylated with *p*-nitrophenyl chloroformate (150 mg, 0.75 mmol) and 2,4,6-trimethylpyridine (0.06 mL, 0.45 mmol) in DCM (2 mL) for 1 h. Next, the resin was washed with DCM, DMF and a solution of DIEA (1 M in DMF, 5 mL) was added to the resin (2x30 min). Finally, after washing the resin with DCM and drying under vacuum, the peptide was cleaved off using the standard cleavage cocktail (5 mL) for 2 h 30 min.



Column A. t = 13.3 min (LYRAF-Dbz), 16.1 min (LYRAF-Nbz), 18.8 min (LYRAF-(*p*NO_2-Phoc)-Dbz).

Calc. $[M+H]^+$: 785.9, found: 785.6.

Figure S2. LYRAF-Nbz. Analytical HPLC trace of the crude synthesis and MALDI-TOF (right) of the compound corresponding to the peak at 16.1 min.

2.3. Synthesis of LYRAXaa$_1$-(*p*CN-Phoc)Dbz and LYRIP-(*p*CN-Phoc)Dbz

LYRAG-(*p*CN-Phoc)Dbz

The following synthesis of LYRAG-(*p*CN-Phoc)Dbz summarizes the preparation of model peptides: the Dbz-PAL-resin (aminomethyl NovaGel, 0.5 g) was prepared according the corresponding protocol mentioned in section 2. Following chain assembly (last amino acid was introduced as Boc-Leu), protected Boc-LY(^{t}Bu)R(Pbf)AG-Dbz-PAL-resin (100 mg) was swollen in DCM. Next, *p*-cyanophenyl chloroformate (60 mg, 0.3 mmol) and 2,4,6-trimethylpyridine (33 µL, 0.25 mmol) dissolved in DCM (2 mL) were added to the resin, and the resulting mixture gently stirred for 1 h. Then, the resin was washed with DCM, DMF and finally the peptide cleaved off the resin using the standard TFA-cocktail cleavage (5 mL) for 2 h 30 min. The workup was carried out similarly to that described in section 2. The recovery crude LYRAG-(*p*CN-Phoc)Dbz was 23 mg (92%).

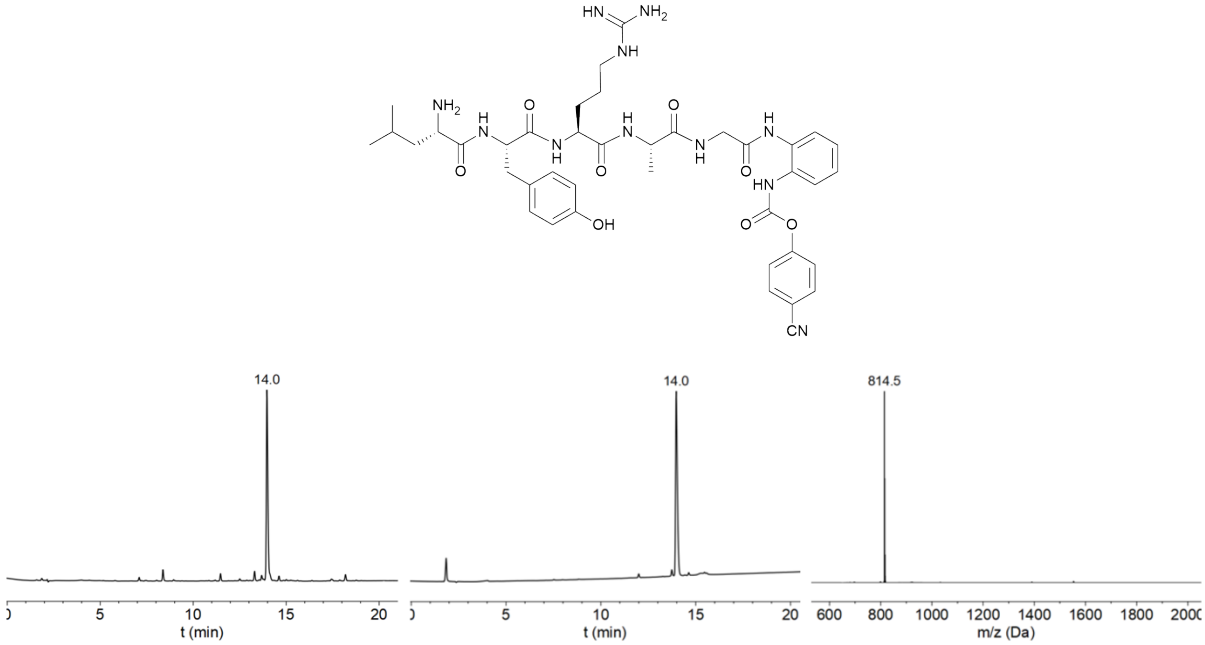
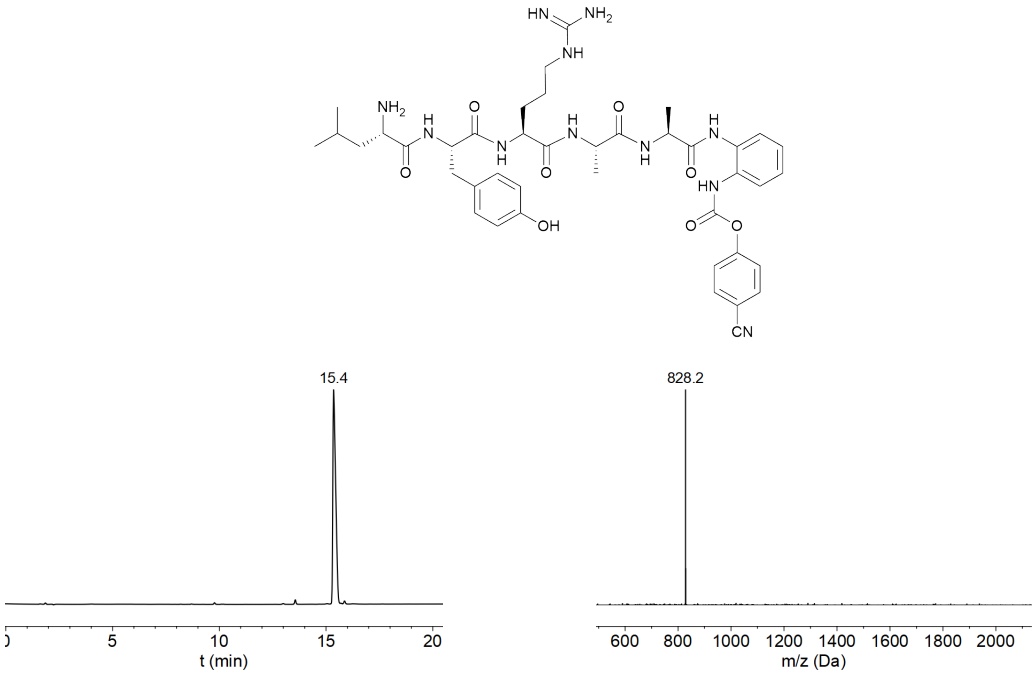


Figure S3. HPLC trace of the crude synthesis of LYRAG-(*p*CN-Phoc)Dbz (left, column A) and the purified peptide (middle). MALDI-TOF of a purified sample of LYRAG-(*p*CN-Phoc)Dbz (right). Calc. $[M+H]^+$: 814.9, found = 814.5.

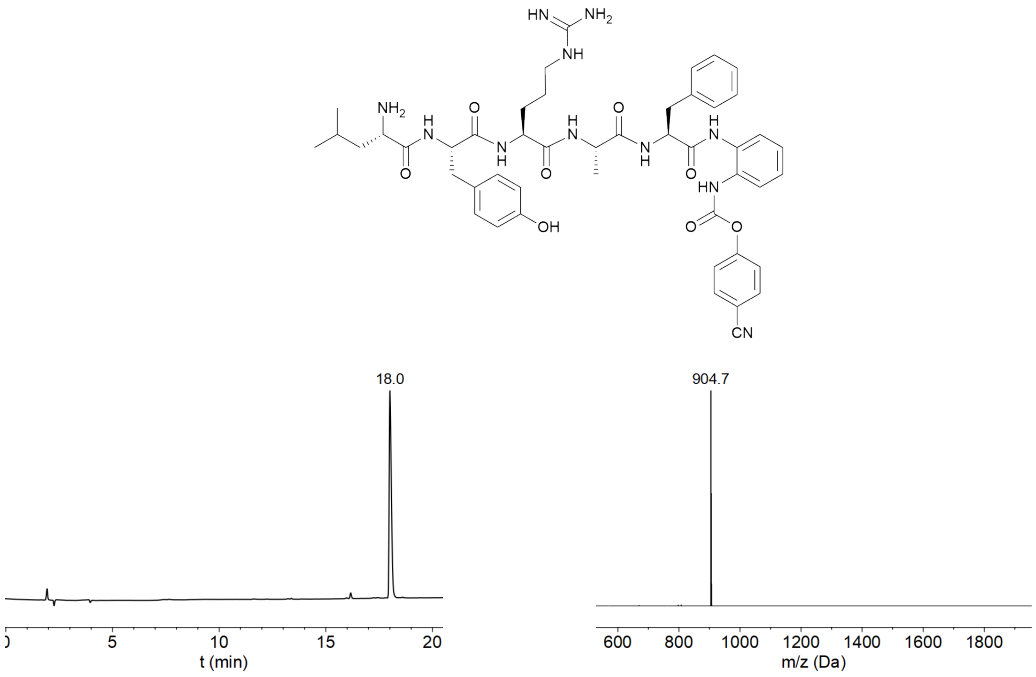
LYRAA-(*p*CN-Phoc)Dbz (12)



Column A. Calc. $[M+H]^+$: 828.9, found: 828.2.

Figure S4. LYRAA-(*p*CN-Phoc)Dbz. Analytical HPLC trace and MALDI-TOF (right) of the purified peptide.

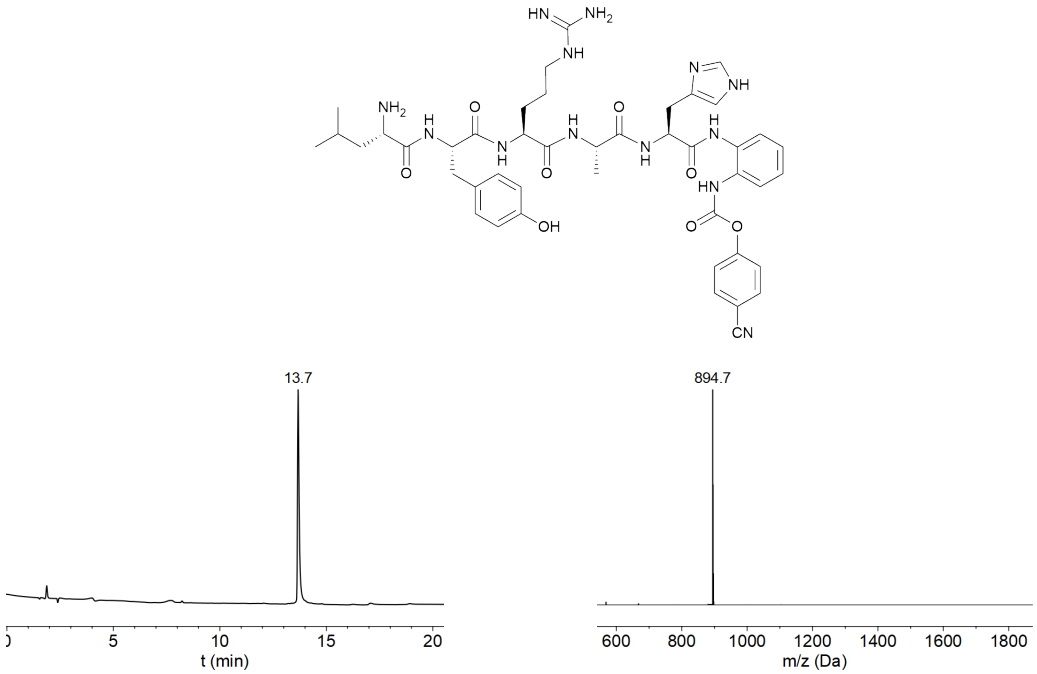
LYRAF-(*p*CN-Phoc)Dbz



Column A. Calc. $[M+H]^+$: 905.0 found: 904.7.

Figure S5. LYRAF-(*p*CN-Phoc)Dbz. Analytical HPLC trace and MALDI-TOF (right) of the purified peptide.

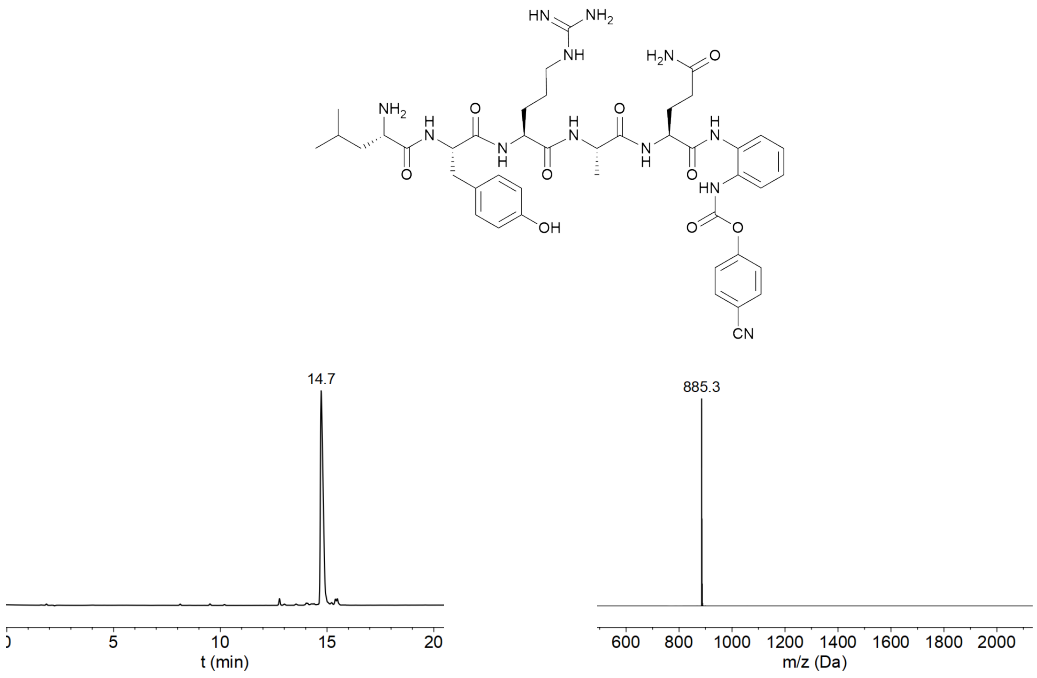
LYRAH-(*p*CN-Phoc)Dbz



Column A. Calc. $[M+H]^+$: 895.0 found: 894.7.

Figure S6. LYRAH-(*p*CN-Phoc)Dbz. Analytical HPLC trace and MALDI-TOF (right) of the purified peptide.

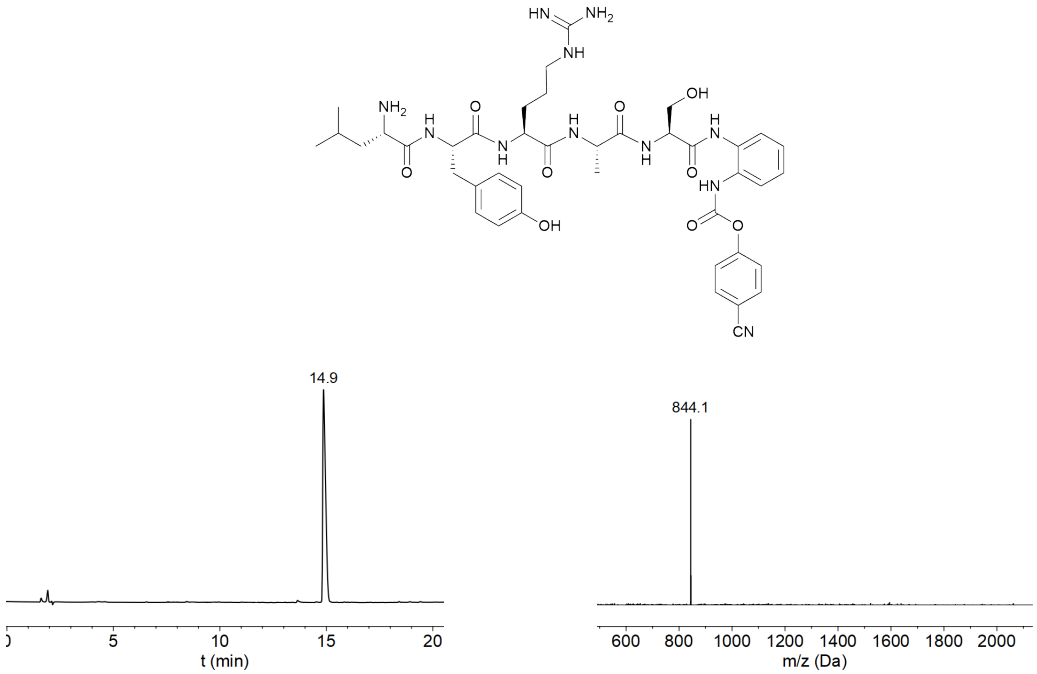
LYRAQ-(*p*CN-Phoc)Dbz



Column A. Calc. $[M+H]^+$: 885.9, found: 885.3.

Figure S7. LYRAQ-(*p*CN-Phoc)Dbz. Analytical HPLC trace and MALDI-TOF (right) of the purified peptide.

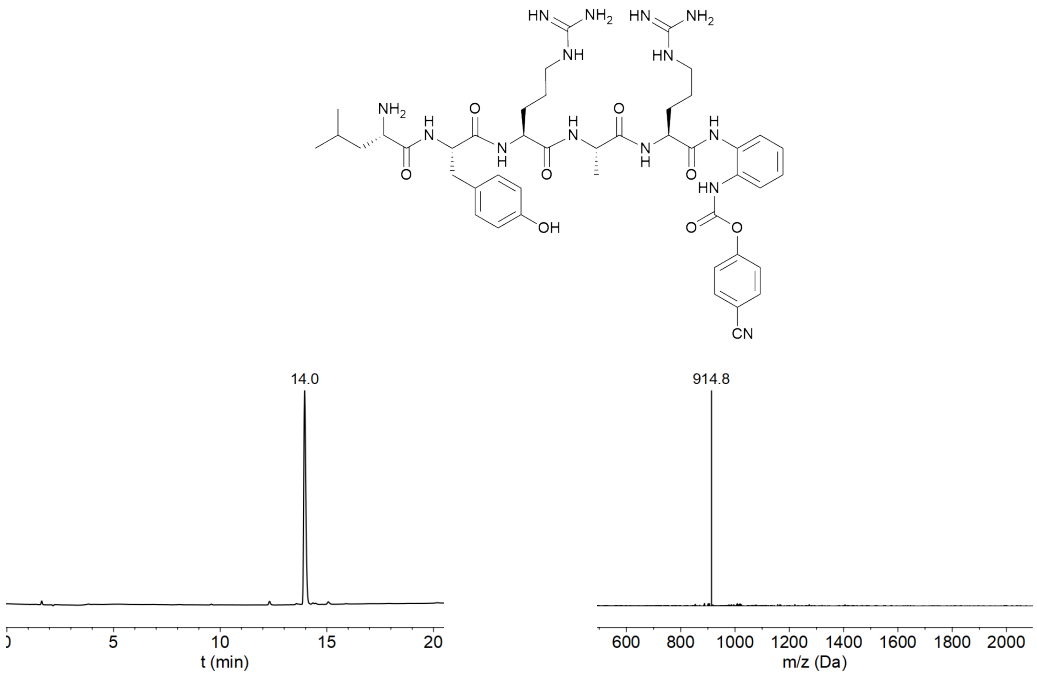
LYRAS-(*p*CN-Phoc)Dbz



Column A. Calc. $[M+H]^+$: 844.4, found: 844.1.

Figure S8. LYRAS-(*p*CN-Phoc)Dbz. Analytical HPLC trace and MALDI-TOF (right) of the purified peptide.

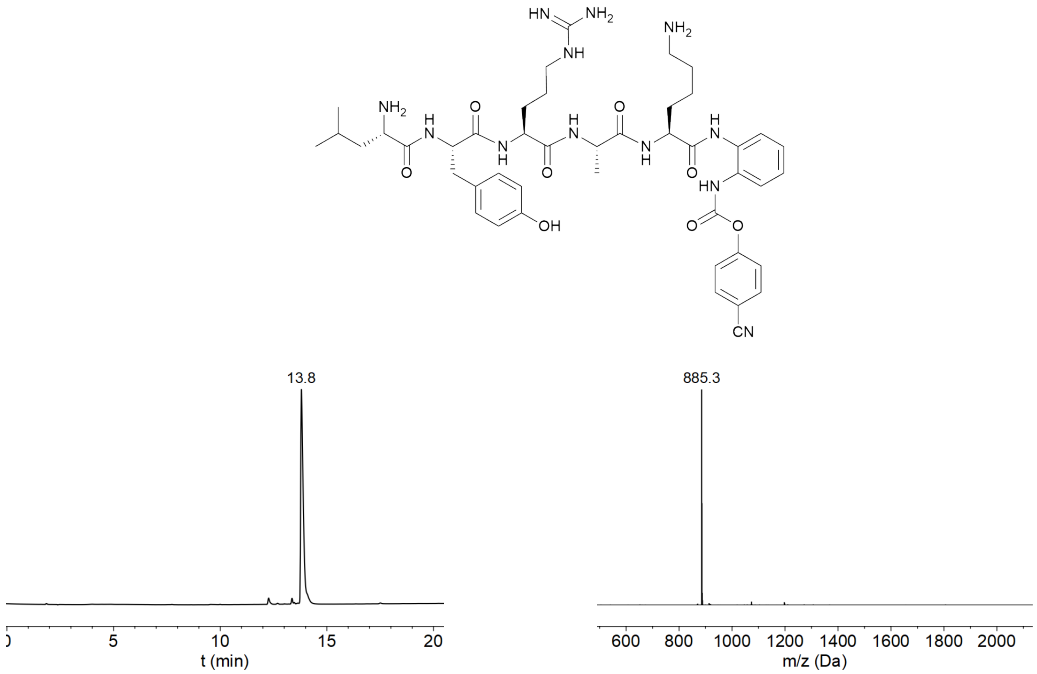
LYRAR-(*p*CN-Phoc)Dbz



Column A. Calc. $[M+H]^+$: 914.1, found: 914.8.

Figure S9. LYRAR-(*p*CN-Phoc)Dbz. Analytical HPLC trace and MALDI-TOF (right) of the purified peptide.

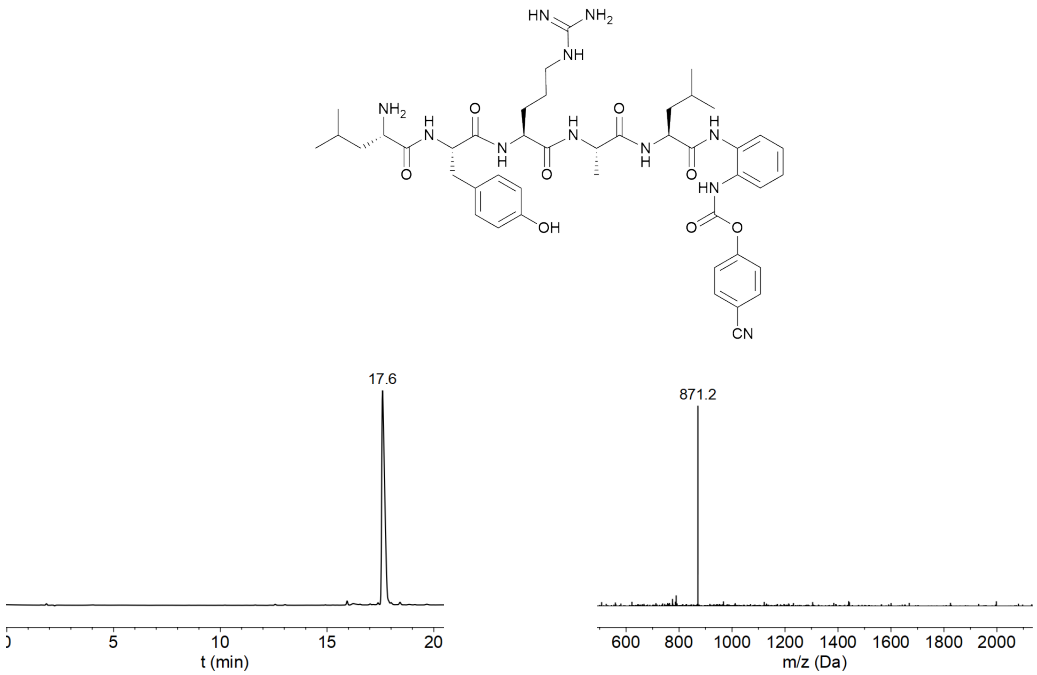
LYRAK-(*p*CN-Phoc)Dbz



Column A. Calc. $[M+H]^+$: 886.0, found: 885.3.

Figure S10. LYRAK-(*p*CN-Phoc)Dbz. Analytical HPLC trace and MALDI-TOF (right) of the purified peptide.

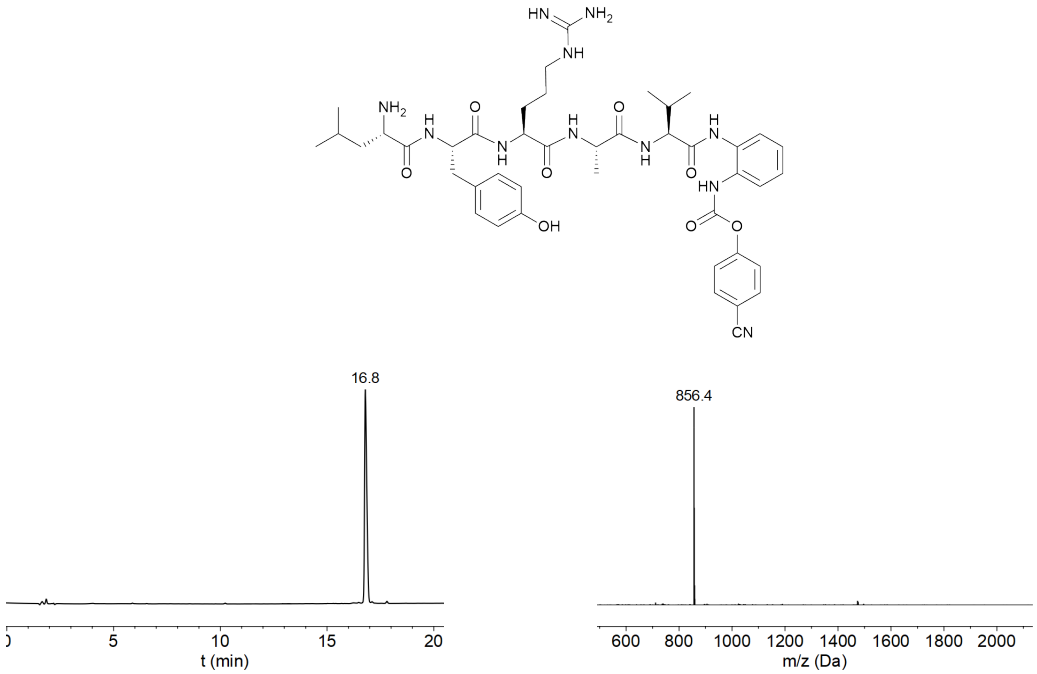
LYRAL-(*p*CN-Phoc)Dbz



Column A. Calc. $[M+H]^+$: 871.0, found: 871.2.

Figure S11. LYRAL-(*p*CN-Phoc)Dbz. Analytical HPLC trace and MALDI-TOF (right) of the purified peptide.

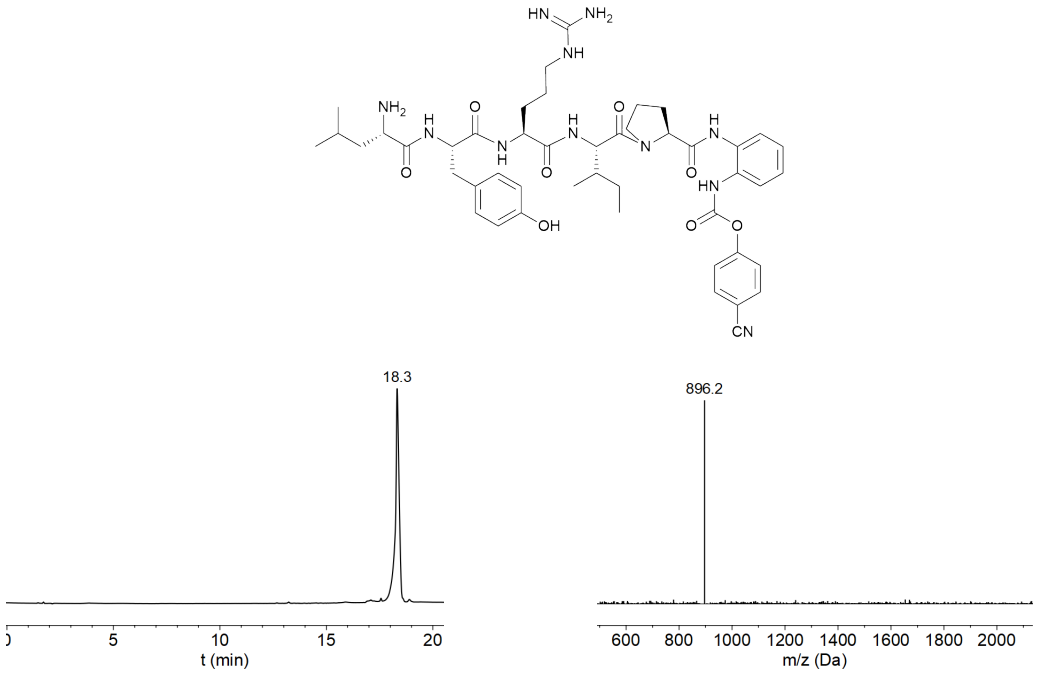
LYRAV-(*p*CN-Phoc)Dbz



Column A. Calc. $[M+H]^+$: 857.0, found: 856.4.

Figure S12. LYRAV-(*p*CN-Phoc)Dbz. Analytical HPLC trace and MALDI-TOF (right) of the purified peptide.

LYRIP-(*p*CN-Phoc)Dbz



Column A. Calc. $[M+H]^+$: 897.1, found: 896.2.

Figure S13. LYRIP-(*p*CN-Phoc)Dbz. Analytical HPLC trace and MALDI-TOF (right) of the purified peptide.

2.4. Synthesis of LYRAXaa$_1$-Dbz and LYRIP-Dbz

LYRAG-Dbz

The following synthesis of LYRAG-Dbz summarizes the preparation of model peptides: protected Boc-LY(^{t}Bu)R(Pbf)AG-Dbz-PAL-resin (aminomethyl NovaGel, 100 mg, section 2.3) was cleaved off the resin using the standard TFA-cocktail cleavage (5 mL) for 1 h 30 min. The workup was carried out similarly to that described in section 2. The recovery crude LYRAG-Dbz was 21 mg (95%).

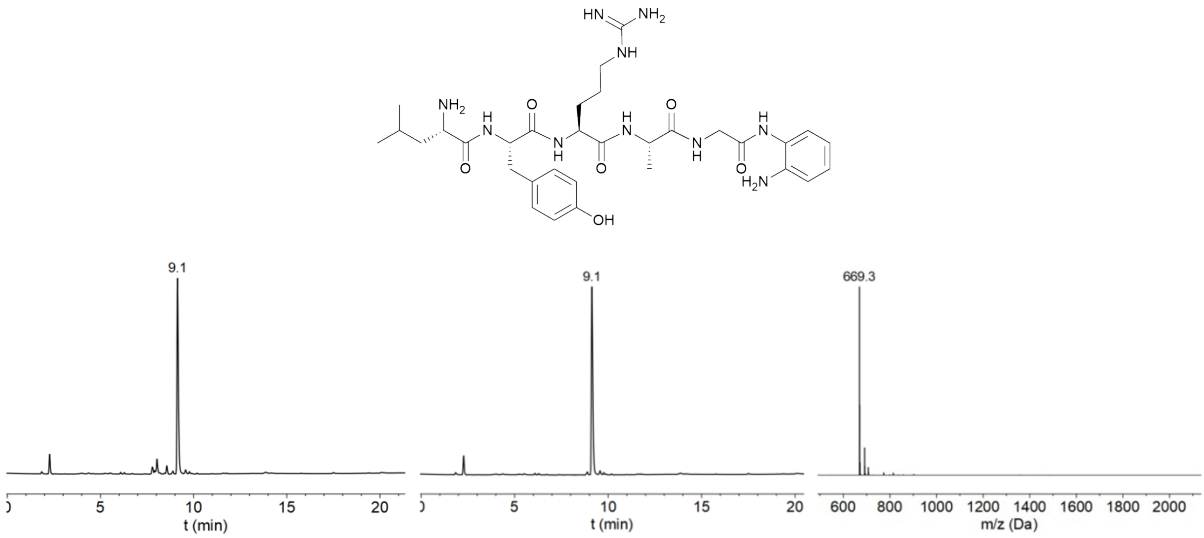
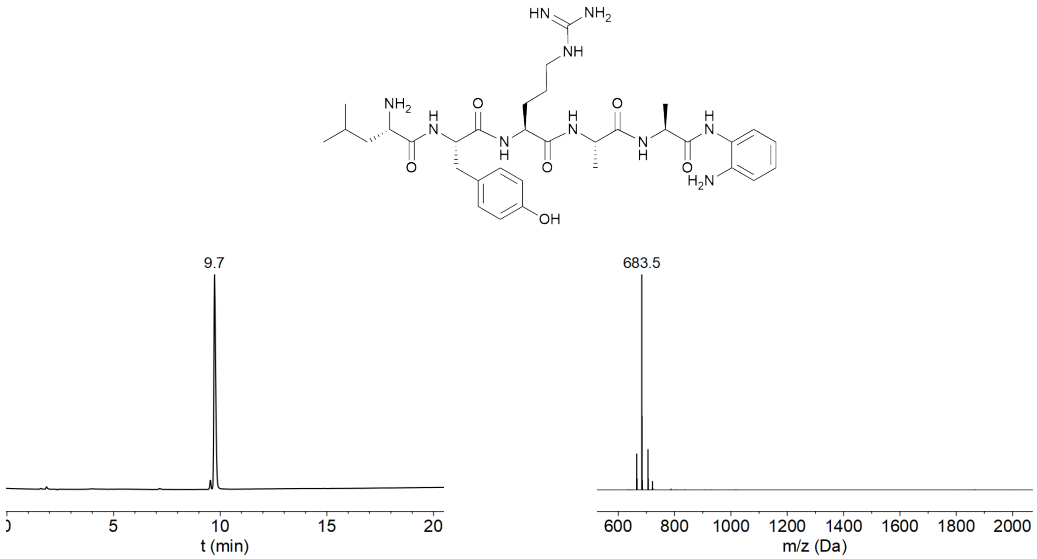


Figure S14. LYRAG-Dbz. HPLC trace of the crude synthesis of LYRAG-Dbz (left, column A) and the purified peptide (middle). MALDI-TOF of a purified sample of LYRAG-Dbz (right). Calc. $[M+H]^+$: 669.8, found = 669.3.

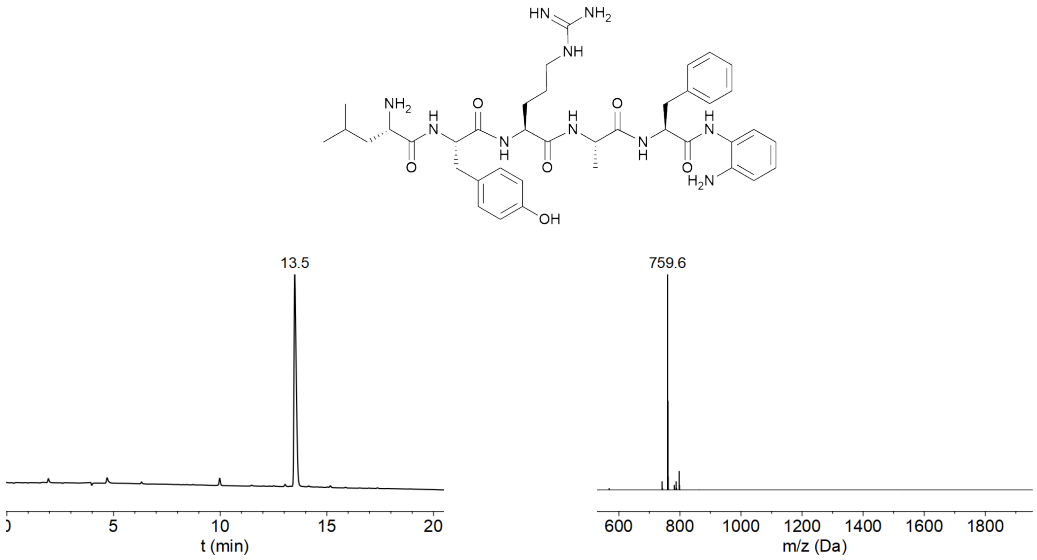
LYRAA-Dbz (25)



Column A. Calc. $[M+H]^+$: 683.8, found: 683.5.

Figure S15. LYRAA-Dbz. Analytical HPLC trace and MALDI-TOF (right) of the purified peptide.

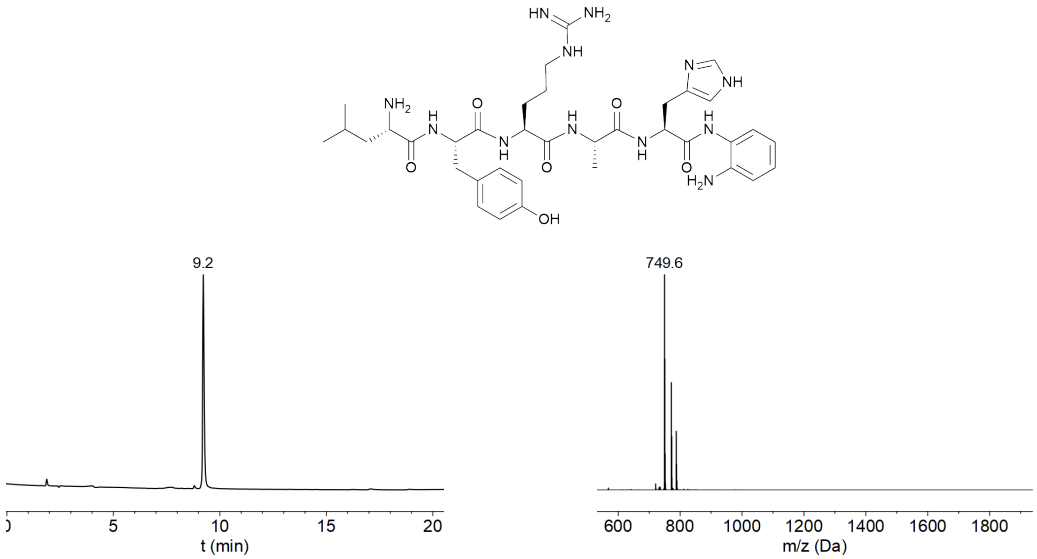
LYRAF-Dbz (29)



Column A. Calc. $[M+H]^+$: 759.9, found: 759.6.

Figure S16. LYRAF-Dbz. Analytical HPLC trace and MALDI-TOF (right) of the purified peptide.

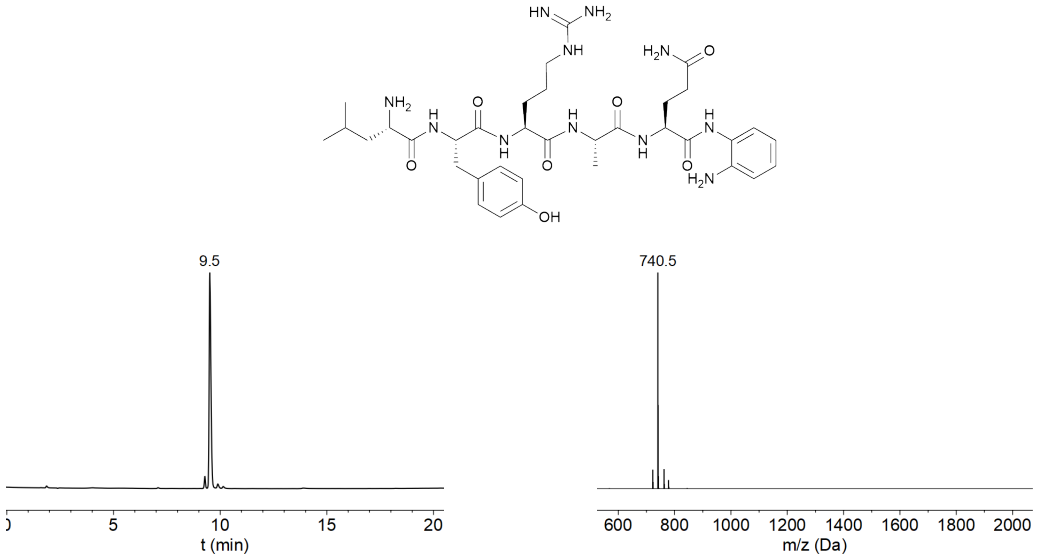
LYRAH-Dbz



Column A. Calc. $[M+H]^+$: 749.9, found: 749.6.

Figure S17. LYRAH-Dbz. Analytical HPLC trace and MALDI-TOF (right) of the purified peptide.

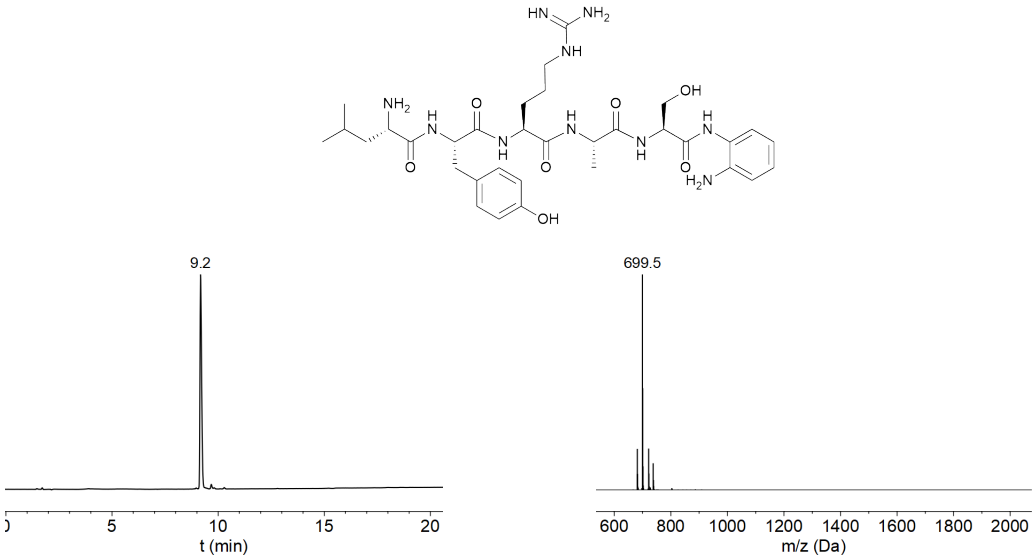
LYRAQ-Dbz



Column A. Calc. $[M+H]^+$: 740.9, found: 740.5.

Figure S18. LYRAQ-Dbz. Analytical HPLC trace and MALDI-TOF (right) of the purified peptide.

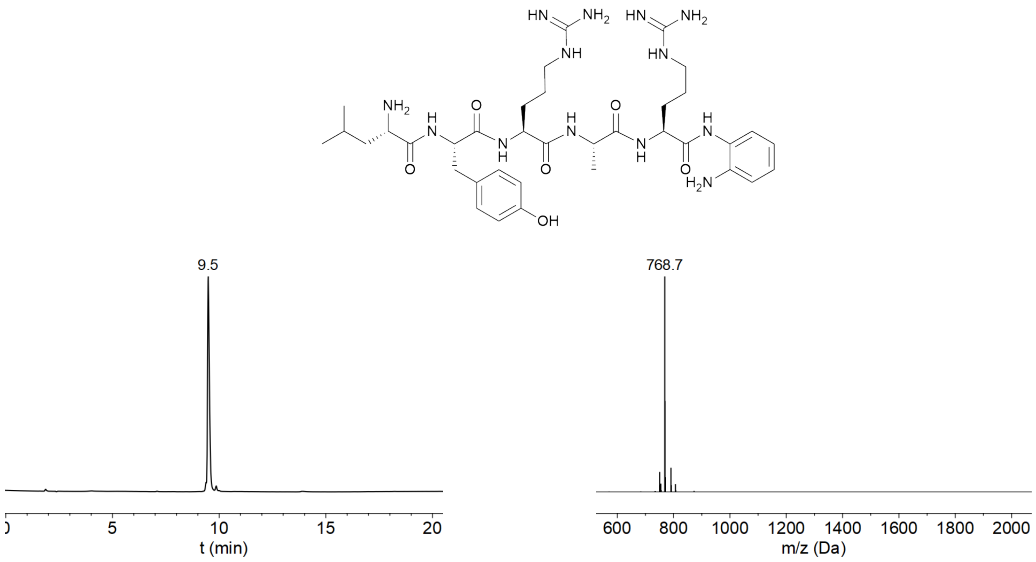
LYRAS-Dbz



Column A. Calc. $[M+H]^+$: 699.8, found: 699.5.

Figure S19. LYRAS-Dbz. Analytical HPLC trace and MALDI-TOF (right) of the purified peptide.

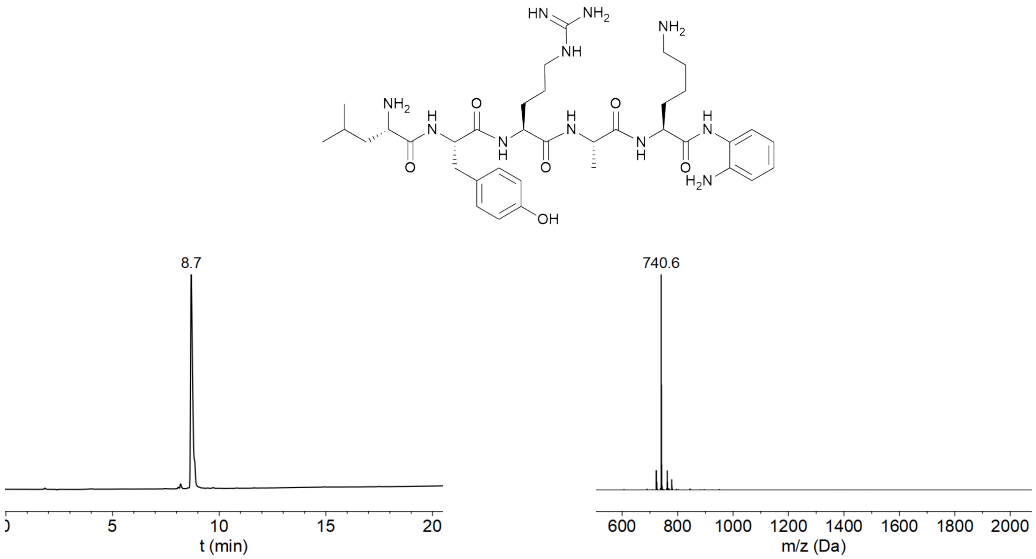
LYRAR-Dbz



Column A. Calc. $[M+H]^+$: 768.9, found: 768.7.

Figure S20. LYRAR-Dbz. Analytical HPLC trace and MALDI-TOF (right) of the purified peptide.

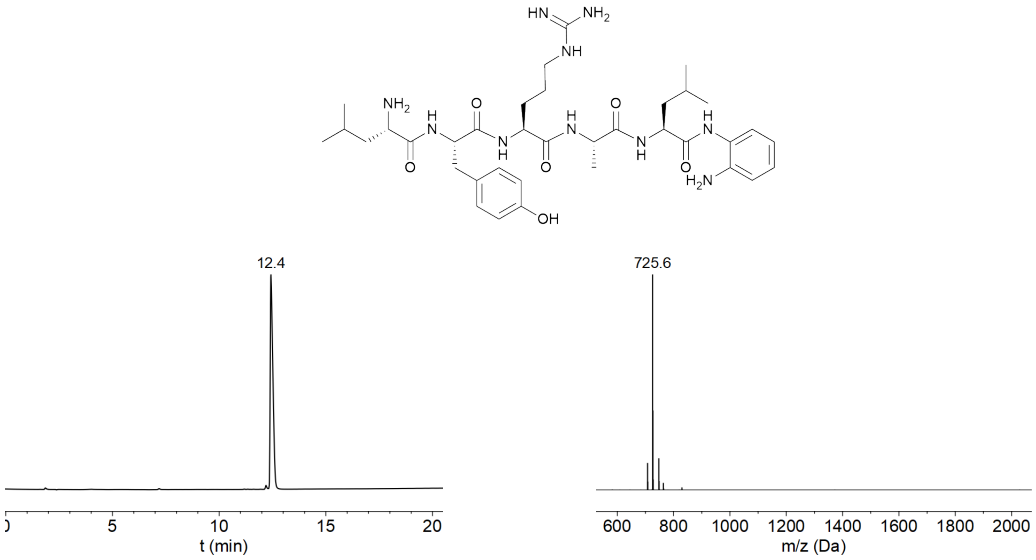
LYRAK-Dbz



Column A. Calc. $[M+H]^+$: 740.9, found: 740.6.

Figure S21. LYRAK-Dbz. Analytical HPLC trace and MALDI-TOF (right) of the purified peptide.

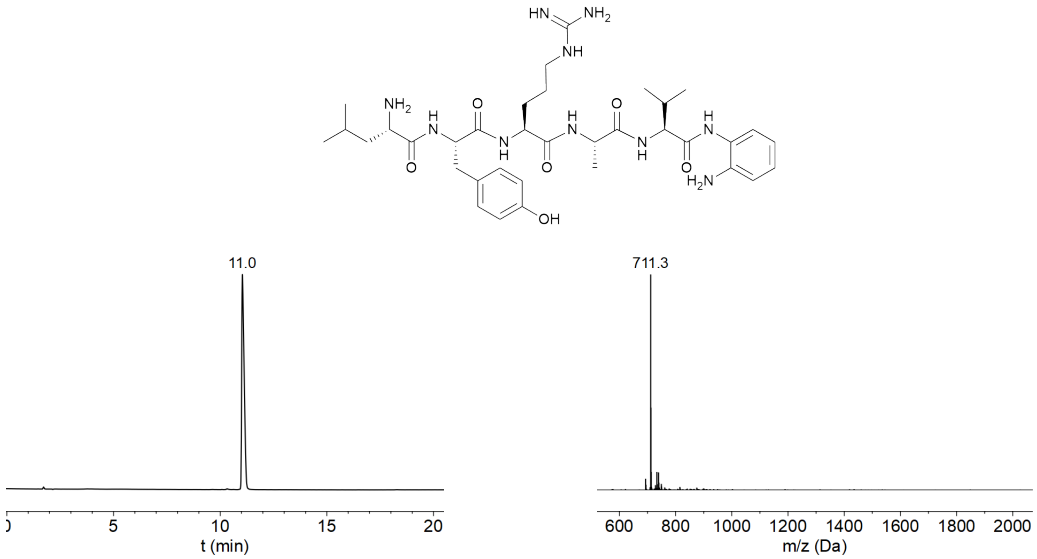
LYRAL-Dbz



Column A. Calc. $[M+H]^+$: 725.9, found: 725.6.

Figure S22. LYRAL-Dbz. Analytical HPLC trace and MALDI-TOF (right) of the purified peptide.

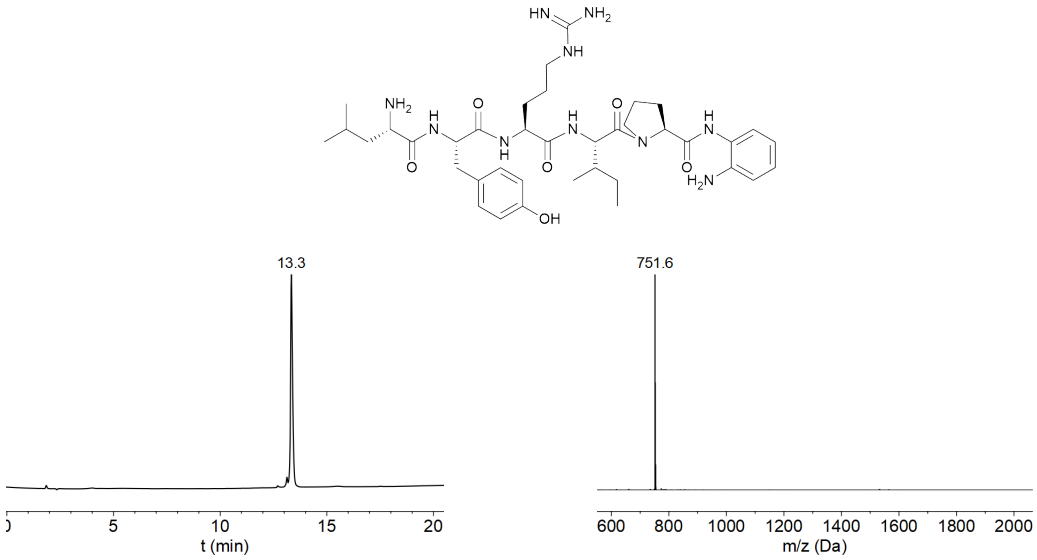
LYRAV-Dbz



Column A. Calc. $[M+H]^+$: 711.8, found: 711.3.

Figure S23. LYRAV-Dbz. Analytical HPLC trace and MALDI-TOF (right) of the purified peptide.

LYRIP-Dbz



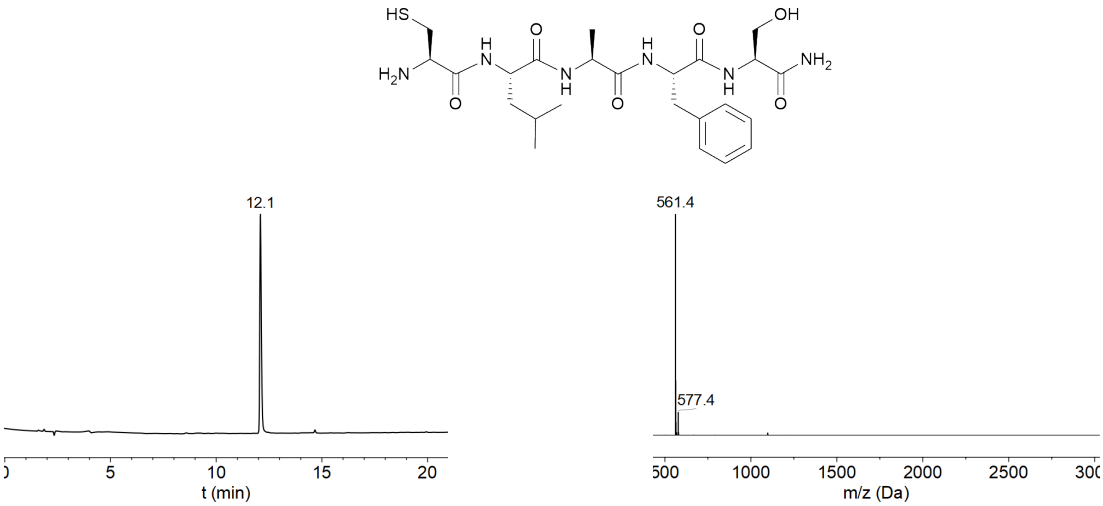
Column A. Calc. $[M+H]^+$: 751.9, found: 751.6.

Figure S24. LYRIP-Dbz. Analytical HPLC trace and MALDI-TOF (right) of the purified peptide.

3. Synthesis of CXaa$_2$AFS peptides

The synthesis was carried out in a Rink-Amide PS resin (0.74 mmol/g).

CLAFS

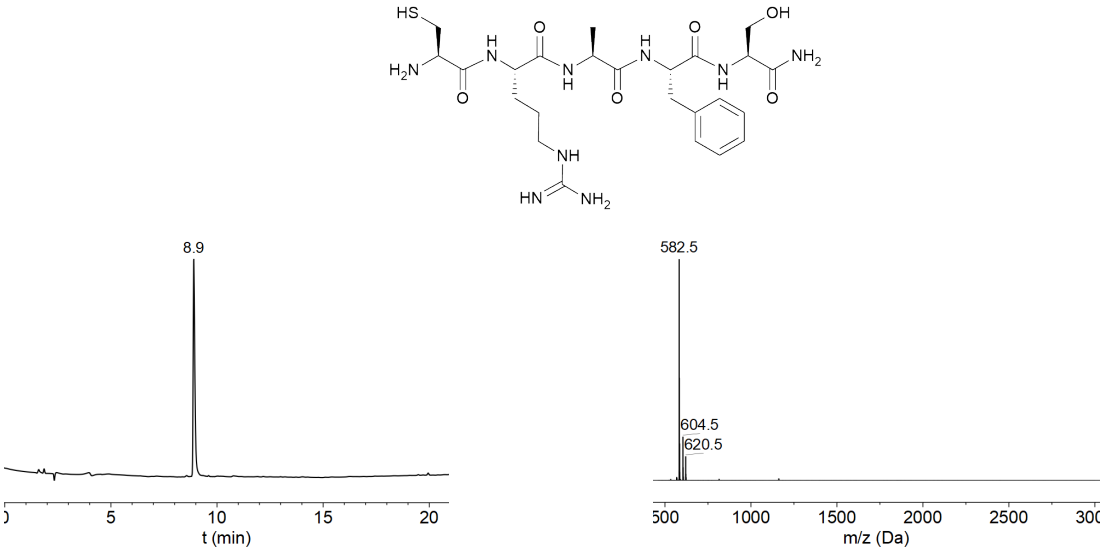


Column A.

Calc. $[M+Na]^+$: 561.6, $[M+K]^+$: 577.5, found: 561.4, 577.4.

Figure S25. CLAFS. Analytical HPLC trace and MALDI-TOF (right) of the purified peptide.

CRAFS (13)

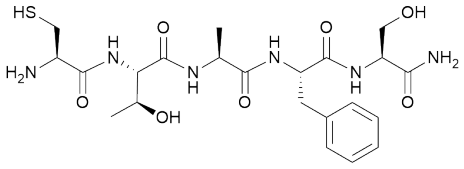


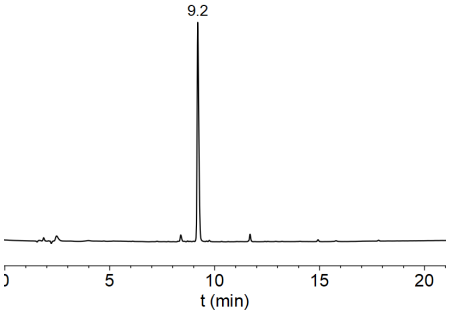
Column A.

Calc. $[M+H]^+$: 582.3, found: 582.5.

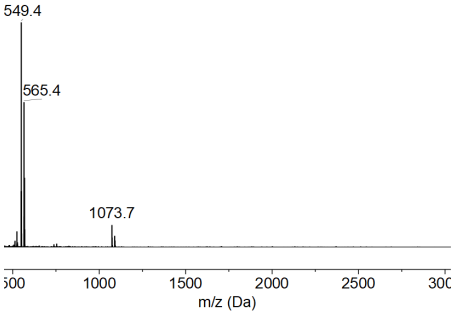
Figure S26. CRAFS. Analytical HPLC trace and MALDI-TOF (right) of the purified peptide.

CTAFS (27)





Column A.



Calc. $[M+Na]^+$: 549.6 $[M+K]^+$: 565.6, found: 549.4, 565.4.

Figure S27. CTAFS. Analytical HPLC trace and MALDI-TOF (right) of the purified peptide.

4. Chemical ligations with peptideXaa$_1$-(*p*CN-Phoc)Dbz

Ligation buffer stock preparation

For NCL experiments, the buffer composition was: Gdm.HCl (6 M), NaPhos (0.2 M), 4-MPOH (200 mM), TCEP.HCl (50 mM) in degassed milli Q H_2O. A stock solution of the buffer was prepared every week and kept at pH = 4.5 under N_2 atmosphere. The final pH of the ligation was adjusted using either a solution of $NaOH_{(aq)}$ 10 M or 1 M, depending of the final fitting and the presence or not of the peptide-thioester (only used 1 M for pH adjusting).

Ligated peptide	PeptideXaa$_1$-(*p*CN-Phoc)Dbz disappearance (min)	PeptideXaa$_1$-(*p*OH-phenylthio-carbonyl)Dbz dissapearance (h)	PeptideXaa$_1$-Nbz dissapearance (h)	Completion (h)	HPLC conversion
LYRAACRAFS	5	1.5	2	2	95%
LYRAGCLAFS	5	0.75	5	5	98%
LYRAFCTAFS	5	2	4	5	95%
LYRAHCTAFS	1	0.5	0.75	0.75	95%
LYRAQCTAFS	5	0.75	1	1.5	95%
LYRASCRAFS	5	1	2	3	90%
LYRARCRAFS	2	1.5	2	2	95%
LYRAKCTAFS	2	2	2	2	90%
LYRALCTAFS	5	3	5	5	98%
LYRAVCTAFS	5	3	23	23	93%
LYRIPCTAFS	5	3	72	72	88%

Table S1. PeptideXaa$_1$-(*p*CN-Phoc)Dbz ligations data.

Data fitting of Figures 2B and 2C (manuscript) was performed using the sigmoidal dose-response (variable slope) of Graphpad Prism: https://www.graphpad.com/guides/prism/latest/curve-fitting/reg_classic_dr_variable.htm

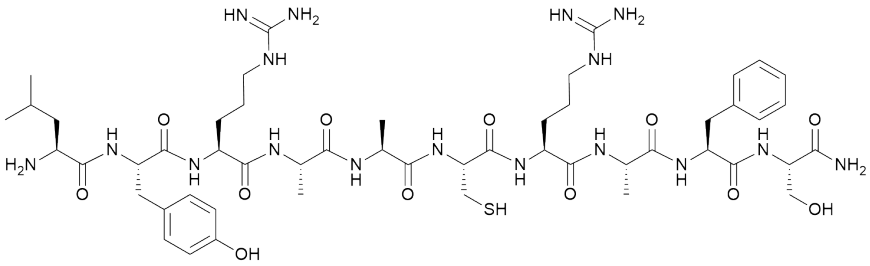
LYRAACRAFS (14)

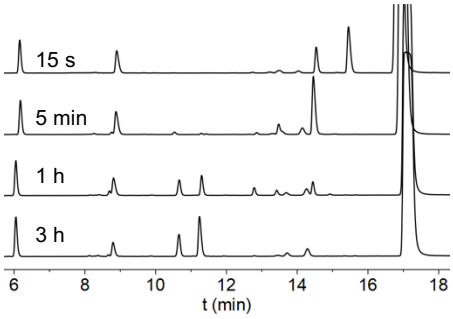
LYRAA-(*p*CN-Phoc)Dbz (1.0 mg, $1.2x10^{-3}$ mmol) and CRAFS (1.0 mg, $1.7x10^{-3}$ mmol) were dissolved in the ligation buffer (0.54 mL, pH = 7.0). A stock solution of L-$Tyr_{(aq)}$ (20 mM, 60 µL, internal standard) was added and the reaction monitored by HPLC by taking aliquots that were quenched with $HCl_{(aq)}$ (0.5 M) at the indicated times.

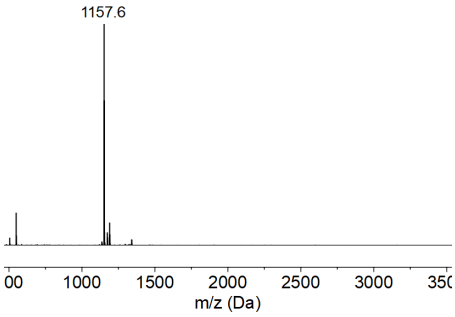
Analysis and product identification of the different HPLC peaks was carried out by MALDI-TOF. The ligated fraction (conversion) was determined based on the area of the product and L-Tyr at 280 nm. The ligated product **14** was found at t = 11.2 min, and characterized by its UV spectrum and MALDI-TOF ($[M+H]^+$: 1157.6). Other products: Nbz (t = 10.6 min), LYRAA-(4-MPOH) (**17**, t = 12.7 min, $[M+H]^+$: 701.3), LYRAA-Nbz (**16**, t = 13.3 min, $[M+H]^+$: 709.3), *p*-cyanophenol (t = 14.3 min) and LYRAA-(*p*OH-phenylthiocarbonyl)Dbz (**15**, t = 14.6 min, $[M+H]^+$: 835.4).

Chemical ligations between other LYRAXaa$_1$-(*p*CN-Phoc)Dbz, LYRIP-(*p*CN-Phoc)Dbz and CXaa$_2$AFS were carried out similarly using an excess of CXaa$_2$AFS (1.5-2 eq).

The following figures show representative chromatograms of the ligations between LYRAXaa$_1$-(*p*CN-Phoc)Dbz, LYRIP-(*p*CN-Phoc)Dbz and CXaa$_2$AFS.

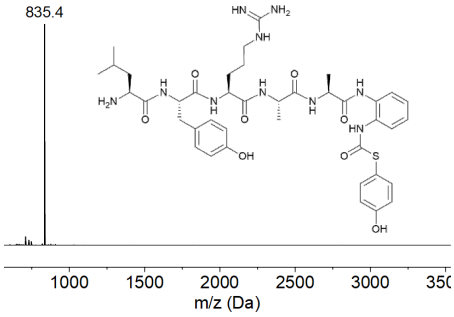




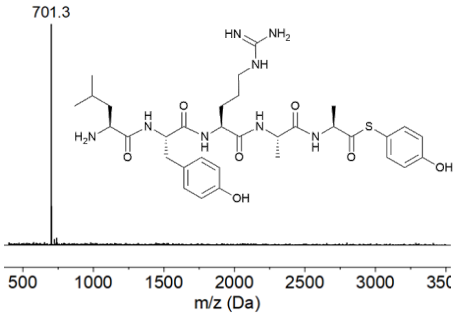


Column B. t = 6.0 min (L-Tyr), 8.7 min (CRAFS), 10.6 min (Nbz), 11.2 min (LYRAACRAFS), 12.7 min (LYRAA-(4-MPOH)), 13.3 min (LYRAA-Nbz), 14.3 min (*p*-cyanophenol), 14.6 min (LYRAA-(*p*OH-phenylthiocarbonyl)Dbz), 15.5 min (LYRAA-(*p*CN-Phoc)Dbz).

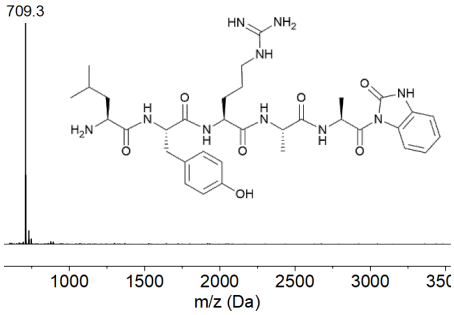
Calc. $[M+H]^+$: 1157.4, found: 1157.6.



Calc. $[M+H]^+$: 836.0, found: 835.4.



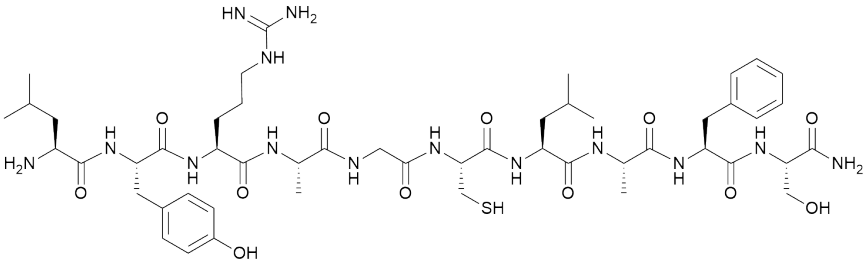
Calc. $[M+H]^+$: 701.9, found: 701.3.

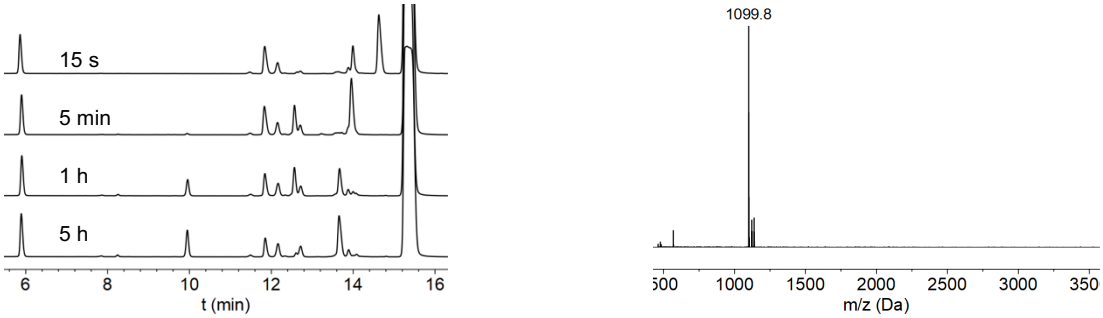


Calc. $[M+H]^+$: 709.9, found: 709.3.

Figure S28. LYRAACRAFS. Analytical HPLC trace of the ligation at time 15 s, 5 min, 1 h and 3 h. MALDI-TOF of the ligation product and different intermediate peptides.

LYRAGCLAFS



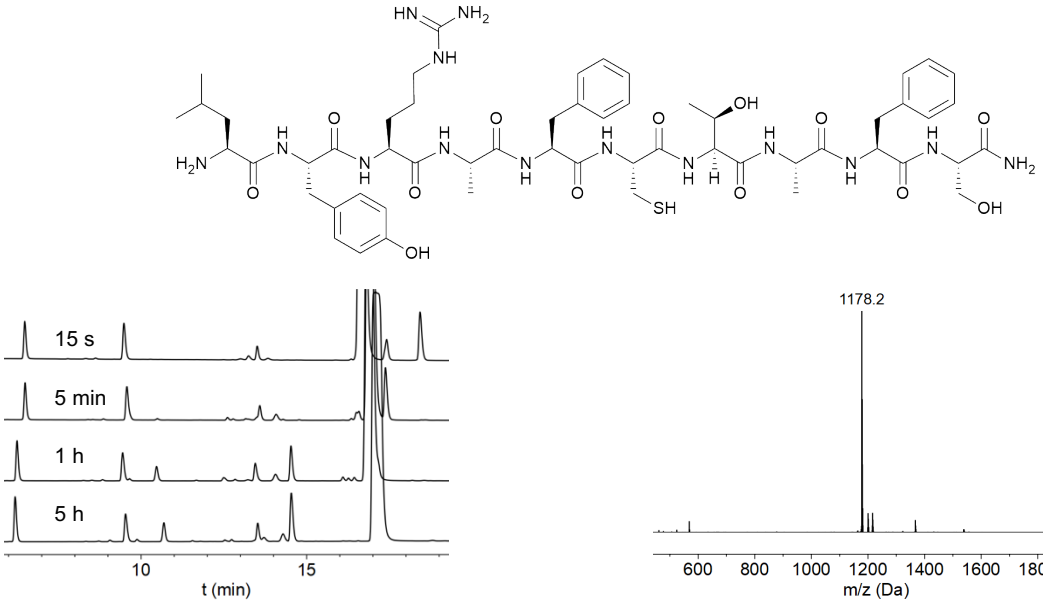


Column A. t = 5.9 min (L-Tyr), 10.0 min (Nbz), 11.9 min (CLAFS), 12.6 min (LYRAG-Nbz), 12.7 min (*p*-cyanophenol), 13.7 min (LYRAGCLAFS), 14.0 min (LYRAG-(*p*OH-phenylthiocarbonyl)Dbz), 14.6 min (LYRAG-(*p*CN-Phoc)Dbz, **20**).

Calc. $[M+H]^+$: 1100.6, found: 1099.8.

Figure S29. LYRAGCLAFS. Analytical HPLC trace of the ligation at time 15 s, 5 min, 1 h and 5 h. MALDI-TOF (right) of the ligation product.

LYRAFCTAFS (32)

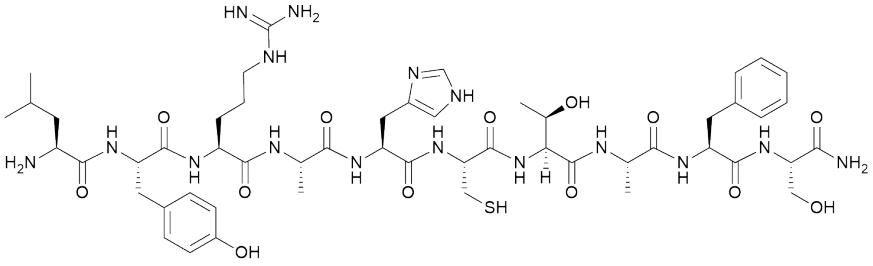


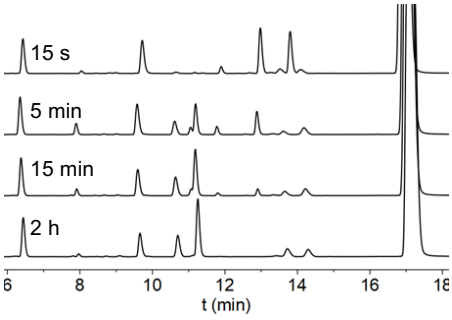
Column B. t = 6.4 min (L-Tyr), 9.6 min (CTAFS), 10.7 min (Nbz), 14.5 min (LYRAFCTAFS), 14.3 min (*p*-cyanophenol), 16.8 (LYRAF-Nbz), 17.5 min (LYRAF-(*p*OH-phenylthiocarbonyl)Dbz), 18.3 min (LYRAF-(*p*CN-Phoc)Dbz).

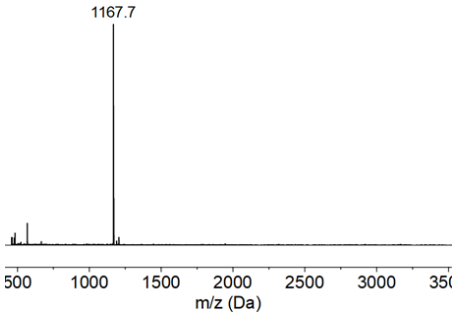
Calc. $[M+H]^+$: 1178.4, found: 1178.2.

Figure S30. LYRAFCTAFS. Analytical HPLC trace of the ligation at time 15 s, 5 min, 1 h and 5 h. MALDI-TOF (right) of the ligation product.

LYRAHCTAFS





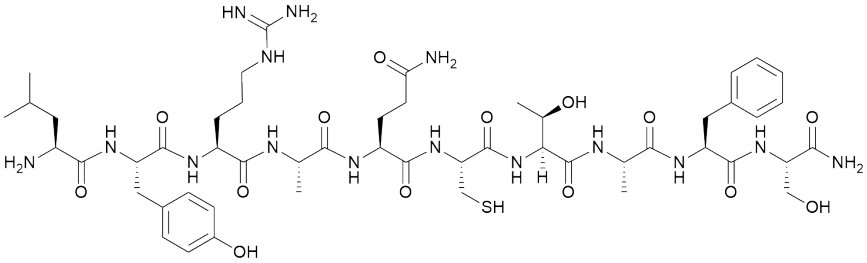


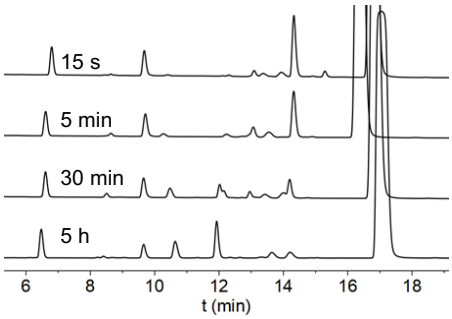
Column B. t = 6.3 min (L-Tyr), 7.9 min (LYRAH-OH), 9.5 min (CTAFS), 10.3 min (Nbz), 10.8 min (LYRAH-(4-MPOH)), 11.1 min (LYRAHCTAFS), 13.0 min (LYRAH-(*p*OH-phenylthiocarbonyl)Dbz), 13.9 min (LYRAH-(*p*CN-Phoc)Dbz), 14.2 min (*p*-cyanophenol).

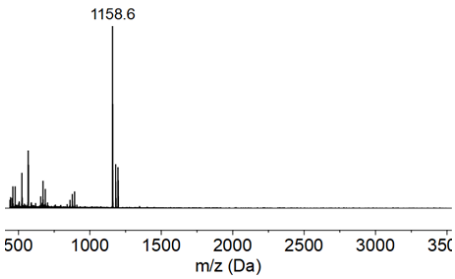
Calc. $[M+H]^+$: 1168.4, found: 1167.7.

Figure S31. LYRAHCTAFS. Analytical HPLC trace of the ligation at time 15 s, 5 min, 15 min and 2 h. MALDI-TOF (right) of the ligation product.

LYRAQCTAFS





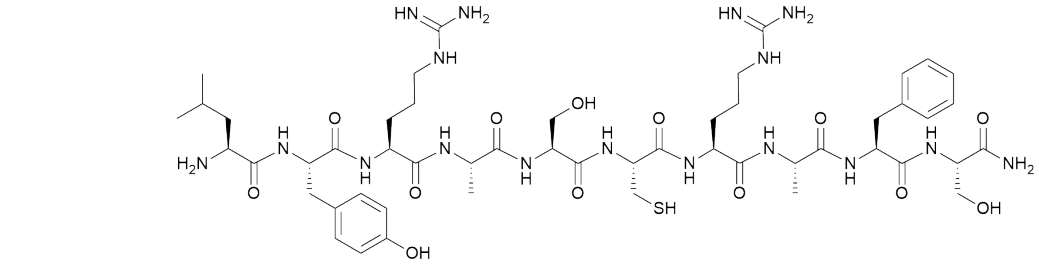


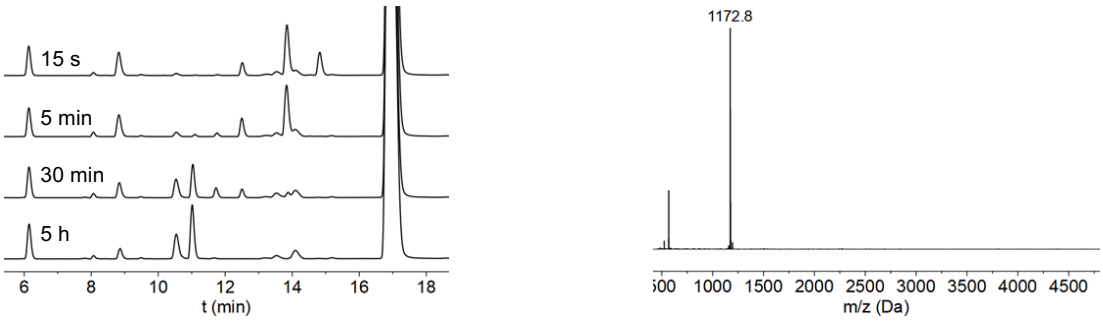
Column B. t = 6.4 min (L-Tyr), 9.5 min (CTAFS), 10.7 min (Nbz), 11.8 min (LYRAQCTAFS), 14.1 min (*p*-cyanophenol), 14.1 min (LYRAQ-(*p*OH-phenylthiocarbonyl)Dbz), 15.3 min (LYRAQ-(*p*CN-Phoc)Dbz).

Calc. $[M+H]^+$: 1158.4, found: 1158.6.

Figure S32. LYRAQCTAFS. Analytical HPLC trace of the ligation at time 15 s, 5 min, 30 min and 5 h. MALDI-TOF (right) of the ligation product.

LYRASCRAFS



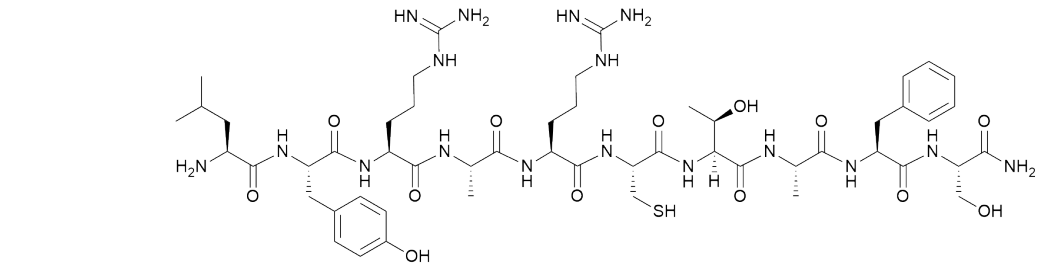


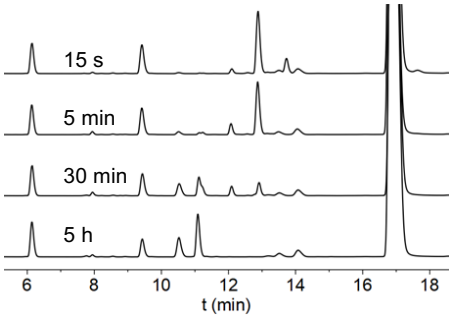
Column B. t = 6.1 min (L-Tyr), 8.9 min (CRAFS), 10.5 min (Nbz), 11.0 min (LYRASCRAFS), 11.8 min (LYRAS-(4-MPOH)), 12.5 min (LYRAS-Nbz), 13.9 min (LYRAS-(*p*OH-phenylthiocarbonyl)Dbz), 14.1 min (*p*-cyanophenol), 14.9 min (LYRAS-(*p*CN-Phoc)Dbz).

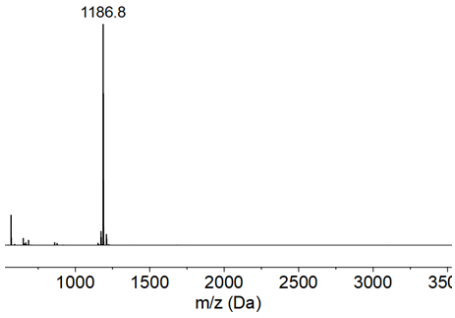
Calc. $[M+H]^+$: 1173.4, found: 1172.8.

Figure S33. LYRASCRAFS. Analytical HPLC trace of the ligation at time 15 s, 5 min, 30 min and 5 h. MALDI-TOF (right) of the ligation product.

LYRARCTAFS





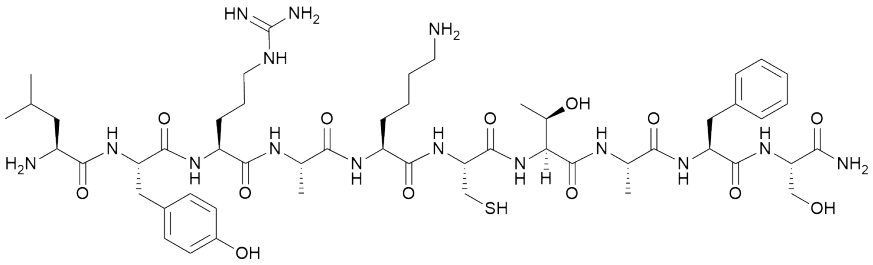


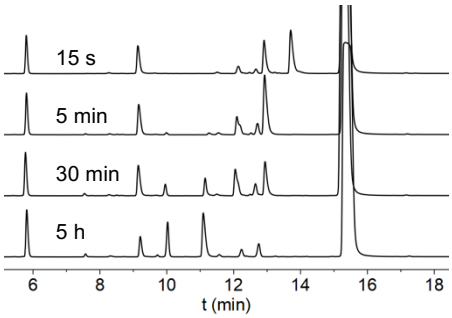
Column B. t = 6.1 min (L-Tyr), 9.4 min (CTAFS), 10.5 min (Nbz), 11.1 min (LYRARCTAFS), 12.8 min (LYRAR-(*p*OH-phenylthiocarbonyl)Dbz), 13.8 min (LYRAR-(*p*CN-Phoc)Dbz), 14.1 min (*p*-cyanophenol).

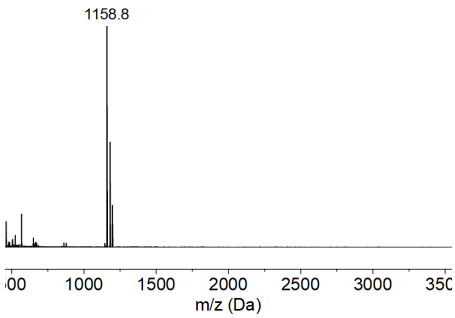
Calc. $[M+H]^+$: 1187.6, found: 1186.8.

Figure S34. LYRARCTAFS. Analytical HPLC trace of the ligation at time 15 s, 5 min, 30 min and 5 h. MALDI-TOF (right) of the ligation product.

LYRAKCTAFS





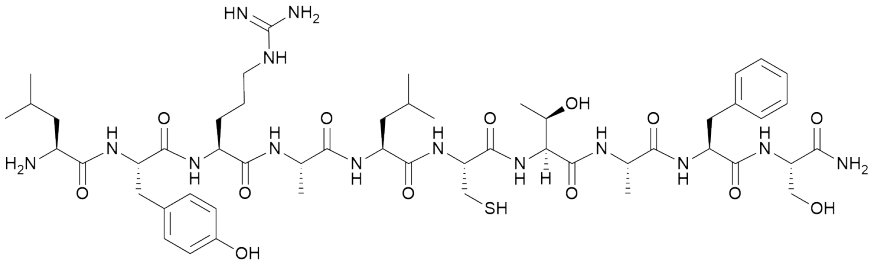


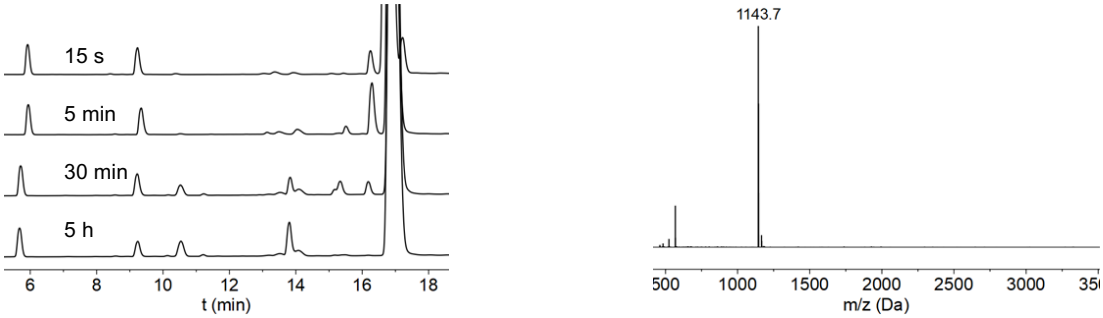
Column A. t = 5.8 min (L-Tyr), 9.2 min (CTAFS), 10.1 min (Nbz), 11.1 min (LYRAKCTAFS), 12.2 min (LYRAK-Nbz), 12.8 min (*p*-cyanophenol), 13.0 min (LYRAK-(*p*OH-phenylthiocarbonyl)Dbz), 13.7 min (LYRAK-(*p*CN-Phoc)Dbz).

Calc. $[M+H]^+$: 1159.6, found: 1158.8.

Figure S35. LYRAKCTAFS. Analytical HPLC trace of the ligation at time 15 s, 5 min, 30 min and 5 h. MALDI-TOF (right) of the ligation product.

LYRALCTAFS



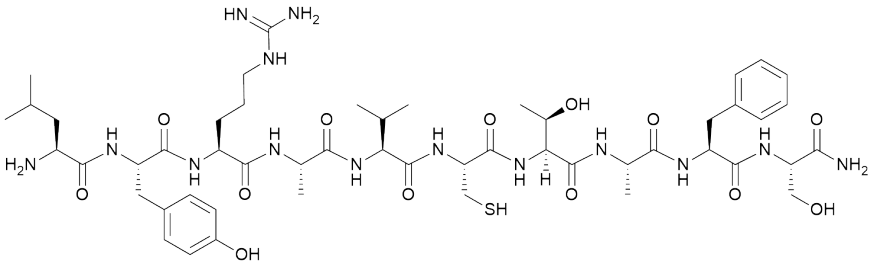


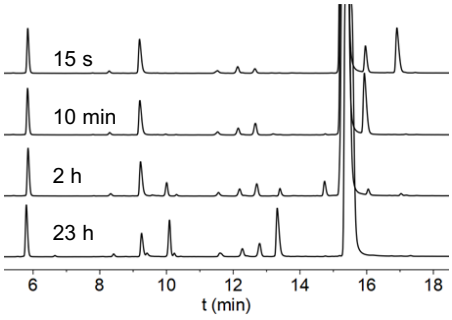
Column B. t = 5.7 min (L-Tyr), 9.2 min (CTAFS), 10.5 min (Nbz), 13.8 min (LYRALCTAFS), 14.2 min (*p*-cyanophenol), 15.2 min (LYRAL-(4-MPOH), 15.5 min (LYRAL-Nbz), 16.3 min (LYRAL-(*p*OH-phenylthiocarbonyl)Dbz), 17.4 min (LYRAL-(*p*CN-Phoc)Dbz).

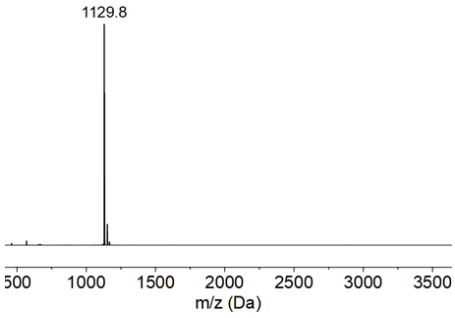
Calc. $[M+H]^+$: 1144.6, found: 1143.7.

Figure S36. LYRALCTAFS. Analytical HPLC trace of the ligation at time 15 s, 5 min, 30 min and 5 h. MALDI-TOF (right) of the ligation product.

LYRAVCTAFS





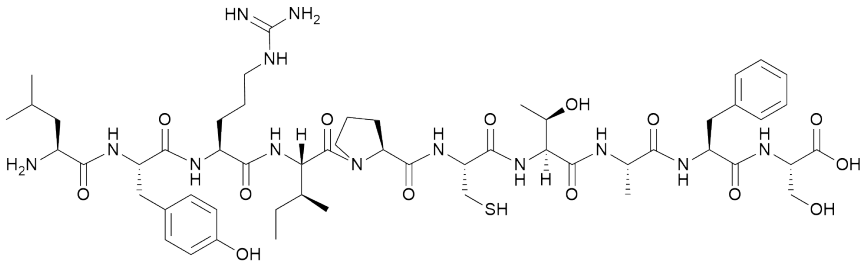


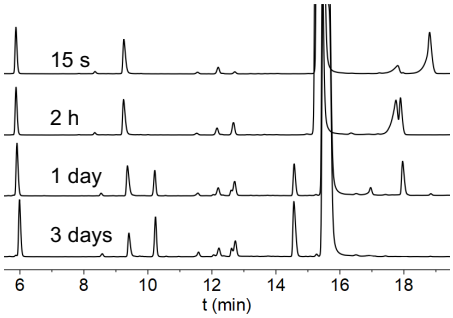
Column A. t = 5.7 min (L-Tyr), 9.1 min (CTAFS), 9.9 min (Nbz), 11.5 min (LYRAV-OH), 12.6 min (*p*-cyanophenol), 13.2 min (LYRAVCTAFS), 14.7 min (LYRAV-Nbz), 16.1 min (LYRAV-(*p*OH-phenylthiocarbonyl)Dbz), 16.9 min (LYRAV-(*p*CN-Phoc)Dbz).

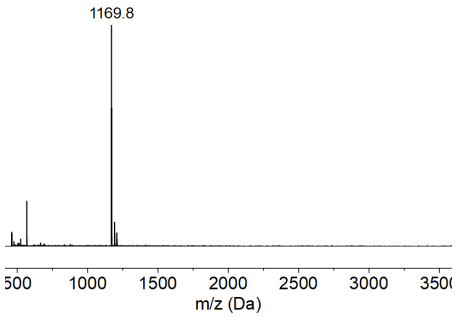
Calc. $[M+H]^+$: 1130.6, found: 1129.8.

Figure S37. LYRAVCTAFS. Analytical HPLC trace of the ligation at time 15 s, 10 min, 2 h and 23 h. MALDI-TOF (right) of the ligation product.

LYRIPCTAFS







Column A. t = 5.8 min (L-Tyr), 9.2 min (CTAFS), 10.0 min (Nbz), 12.6 min (LYRIP-OH), 12.7 min (*p*-cyanophenol), 14.3 min (LYRIPCTAFS), 16.7 min (LYRIP-(4-MPOH)), 17.7 min (LYRIP-(*p*OH-phenylthiocarbonyl)Dbz), 17.8 min (LYRIP-Nbz), 18.6 min (LYRIP-(*p*CN-Phoc)Dbz).

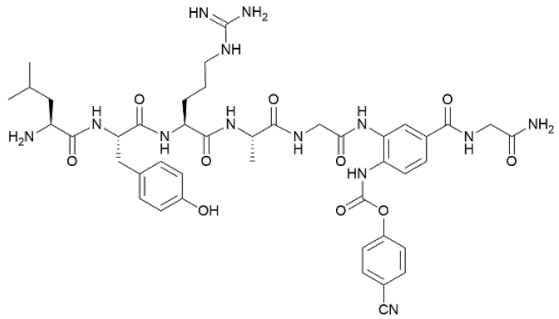
Calc. $[M+H]^+$: 1170.6, found: 1169.8.

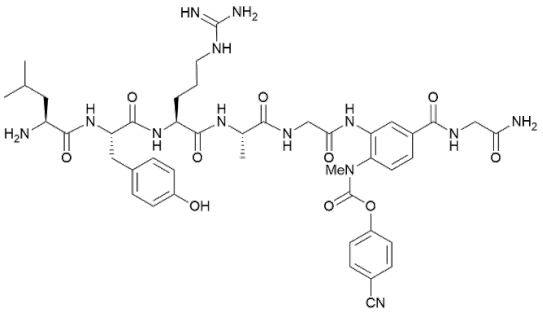
Figure S38. LYRIPCTAFS. Analytical HPLC trace of the ligation at time 15 s, 2 h, 1 d and 3 d. MALDI-TOF (right) of the ligation product.

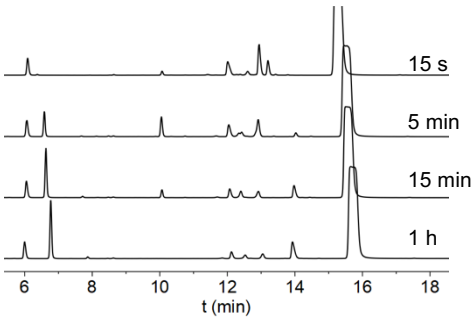
5. Chemical ligations with LYRAG-(*p*CN-Phoc)Dbz-CONH-G (18) and LYRAG-(*p*CN-Phoc)MeDbz-CONH-G (19)

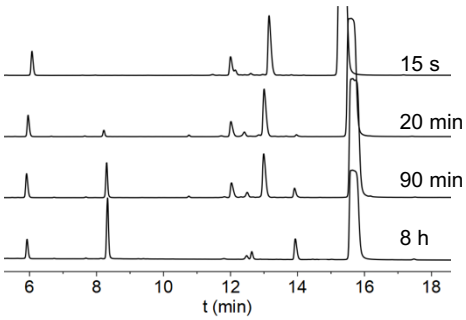
Peptides LYRAG-(*p*CN-Phoc)Dbz-CONH-G (**18**, 1 mg, $1.1x10^{-3}$ mmol) and LYRAG-(*p*CN-Phoc)MeDbz-CONH-G (**19**, 1 mg, $1.1x10^{-3}$ mmol) were ligated in two reactions in the presence of CLAFS (1 mg, $1.7x10^{-3}$ mmol). Peptides were dissolved in the ligation buffer (0.6 mL, pH = 6.85) containing L-Tyr (2 mM). Aliquots of the reactions were withdrawn at the indicated times, quenched with $HCl_{(aq)}$ (0.5 M) and monitored by HPLC.

Analysis and product identification of the different HPLC peaks was carried by MALDI-TOF. The fraction ligated (conversion) was determined based on the area of the product and L-Tyr at 280 nm. The results were plotted for comparison with the ligation of LYRAG-(*p*CN-Phoc)Dbz (**20**, Fig. 1A manuscript).









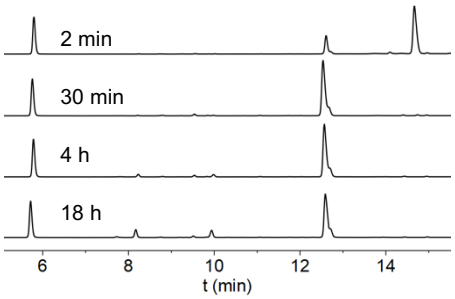
Column A. t = 5.9 min (L-Tyr), 6.7 min (Nbz-CONH-G), 10.0 min (LYRAG-Nbz-CONH-G), 12.1 min (CLAFS), 12.7 min (*p*-cyanophenol), 13.1 min (LYRAG-(*p*OH-phenylthiocarbonyl)Dbz)), 13.5 min (**18**), 14.0 min (LYRAGCLAFS).

Column A. t = 5.9 min (L-Tyr), 8.4 min (MeNbz-CONH-G), 12.1 min (CLAFS), 12.7 min (*p*-cyanophenol), 13.0 min (**19**), 14.0 min (LYRAGCLAFS).

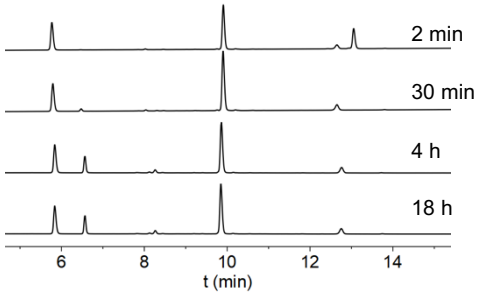
Figure S39. HPLC traces of the chemical ligation between **18** and CLAFS (left), and **19** and CLAFS (right) at the indicated times.

6. Stability of LYRAG-(*p*CN-Phoc)Dbz-CONH-G (18) and LYRAG-(*p*CN-Phoc)Dbz (20) at pH = 7

LYRAG-(*p*CN-Phoc)Dbz-CONH-G (**18**, 1 mg, 1.1×10^{-3} mmol) and LYRAG-(*p*CN-Phoc)Dbz (**20**, 1 mg, 1.1×10^{-3} mmol) were dissolved separately in degassed buffer (6 M Gdm.HCl, 0.2 M NaPhos, 0.6 mL, pH = 7.0) containing L-Tyr (2 mM). The stability of the peptides was monitored by direct injection of aliquots (not quenched) from the solutions every 30 min. Analysis and product identification of the different HPLC peaks was carried out by MALDI-TOF. To calculate the ratio of remaining LYRAG-Nbz-CONH-G and LYRAG-Nbz, peak areas (220 nm) at the selected time points were normalized to the area at 30 min (t_0, 100%): full conversion of **18** to LYRAG-Nbz-CONH-G and **20** to LYRAG-Nbz.



Column A. t = 5.7 min (L-Tyr), 8.1 min (LYRAG-OH), 9.9 min (Nbz), 12.5 min (LYRAG-Nbz), 12.7 min (*p*-cyanophenol), 14.6 min (**20**).



Column A. t = 5.7 min (L-Tyr), 6.6 min (Nbz-CONH-G), 8.1 min (LYRAG-OH), 10.0 min (LYRAG-Nbz-CONH-G), 12.7 min (*p*-cyanophenol), 13.1 min (**18**).

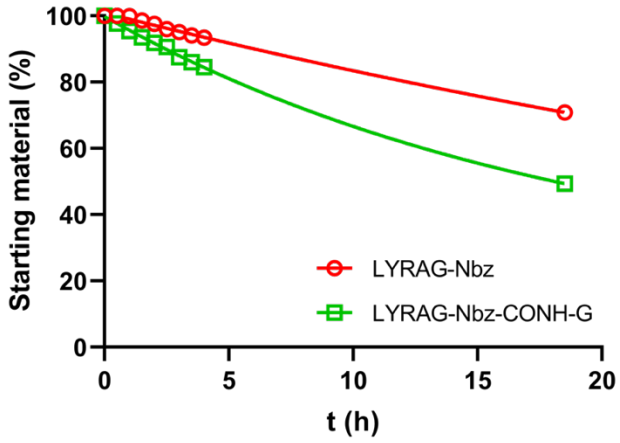


Figure S40. HPLC monitoring of the stability of **20** (left) and **18** (right). Bottom: representation of the ratio of remaining LYRAG-Nbz (red) and LYRAG-Nbz-CONH-G (green) as a function of time.

7. Chemical ligations with peptideXaa_1-Dbz

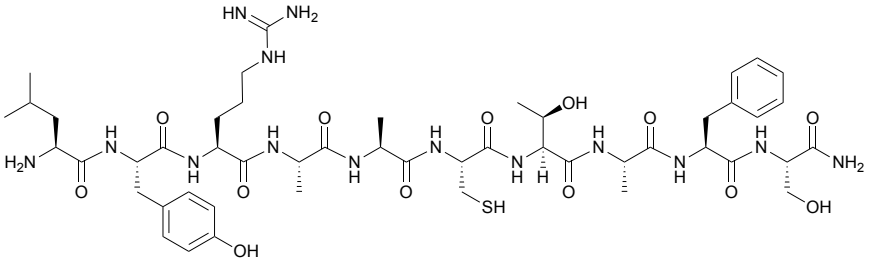
7.1. Stepwise ligation of LYRAA-Dbz

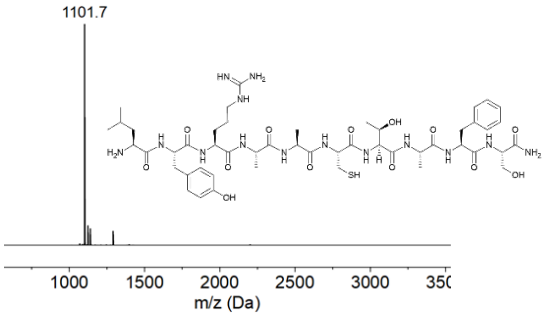
Peptide LYRAA-Dbz (**25**, 1 mg, $1.5x10^{-3}$ mmol) was dissolved in degassed buffer (6 M Gdm.HCl, 0.2 M NaPhos, 0.19 mL, pH = 3). The resulting solution was cooled down in an ice-salt bath (~ –15 °C) and $NaNO_2$ (10 µL of a 0.5 M stock freshly prepared in Gdm buffer, $5x10^{-3}$ mmol) were added. The reaction was kept at ~ –15 °C for 30 min. Then, an aliquot was withdrawn, quenched with the same volume of $HCl_{(aq)}$ (0.5 M) and injected in HPLC (LYRAA-Bt (**26**), Figure 3i, manuscript).

In parallel, a solution of ligation buffer (6 M Gdm.HCl, 0.2 M NaPhos, 200 mM 4-MPOH, 50 mM TCEP.HCl, 0.4 mL, pH = 7.1) containing L-Tyr (3 mM) was added to the formed peptide **26** in the ice-salt bath, and the resulting mixture (final pH = 6.7) left at room temperature for additional 5 min. Next, an aliquot of the reaction was withdrawn, quenched with the same volume of $HCl_{(aq)}$ (0.5 M) and injected in HPLC (LYRAA-(4-MPOH) (**17**), Figure 3ii, manuscript).

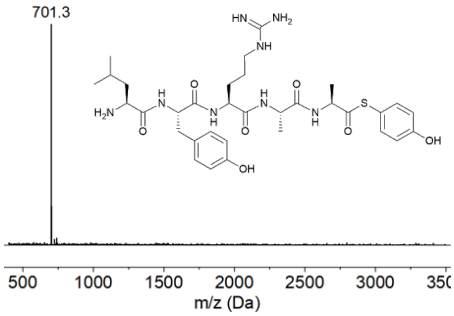
To the above mixture, CTAFS (**27**, 1.5 mg, $2.8x10^{-3}$ mmol) was added and the reaction left (occasional vortexing) at room temperature for an additional 1 h. An aliquot was withdrawn, quenched with the same volume of $HCl_{(aq)}$ (0.5 M) and injected in HPLC (LYRAACTAFS **28**, Figure 3iii, manuscript).

LYRAACTAFS (28)

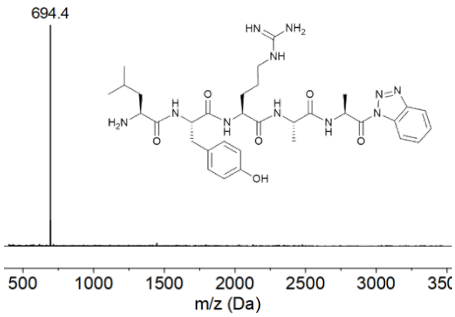




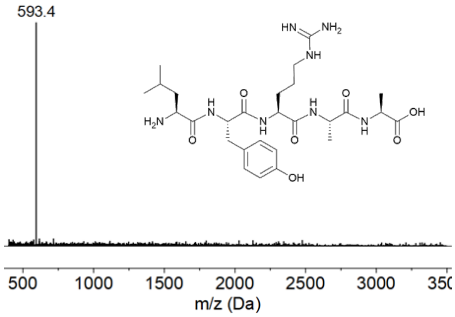
t = 11.8 min (**28**). Calc. $[M+H]^+$: 1101.3, found: 1101.7.



t = 12.5 min (**17**). Calc. $[M+H]^+$: 701.9, found: 701.3.



t = 13.8 min (**26**). Calc. $[M+H]^+$: 694.8, found: 694.4.



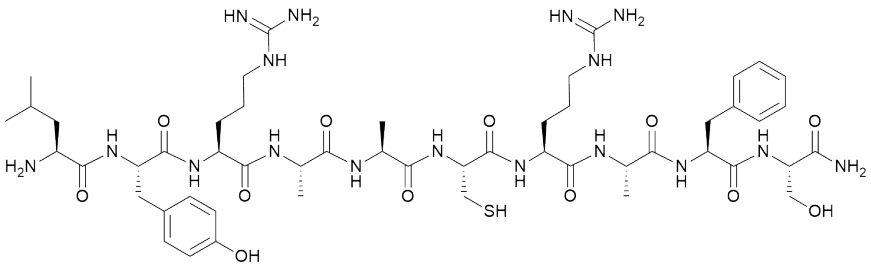
t = 8.6 min. Calc. $[M+H]^+$: 593.7, found: 593.4.

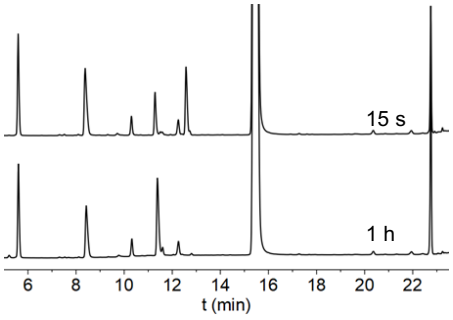
Figure S41. MALDI-TOF analysis of the different products obtained during the stepwise ligation between **25** and **27**.

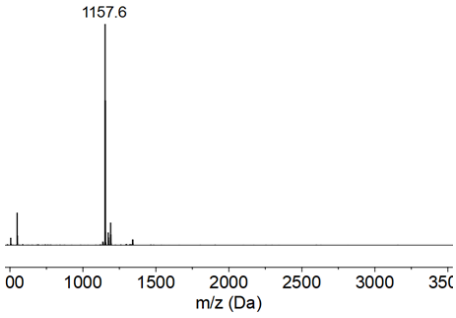
7.2. Two-step one-pot ligation of LYRAA-Dbz

LYRAA-Dbz (**25**, 1 mg, $1.5x10^{-3}$ mmol) and CRAFS (**13**, 1.5 mg, $2.6x10^{-3}$ mmol) were dissolved in degassed buffer (6 Gdm.HCl, 0.2 M NaPhos, 0.19 mL, pH = 3.0) containing L-Tyr (6 mM). The solution was cooled down in an ice-salt bath (~ –15 °C) and $NaNO_2$ (10 µL of a 0.5 M stock freshly prepared in Gdm buffer, $5x10^{-3}$ mmol) was added. The reaction was kept at ~ –15 °C for 30 min. Next, a solution of ligation buffer (0.4 mL, pH = 7.1) was added to the mixture (final pH = 6.7) and the ligation stirred at room temperature for 1 h. The reaction was monitored by HPLC by taking aliquots that were quenched with $HCl_{(aq)}$ (0.5 M) at the indicated times. Peptide identification was carried out by MALDI-TOF analysis of the products corresponding to the different peaks.

Chemical ligations between other LYRAXaa$_1$-Dbz, LYRIP-Dbz and CXaa$_2$AFS were carried out similarly using an excess of CXaa$_2$AFS (1.5-2 eq). Analytical HPLC was run in the column A.





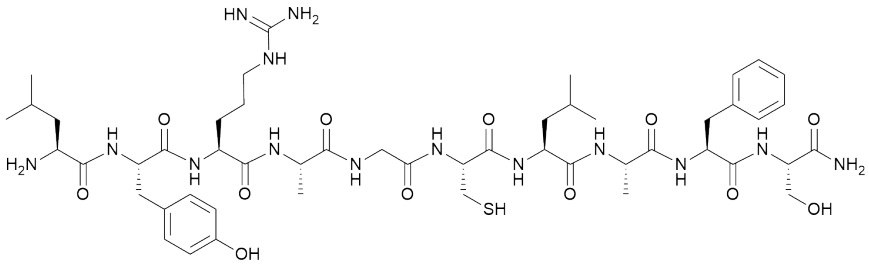


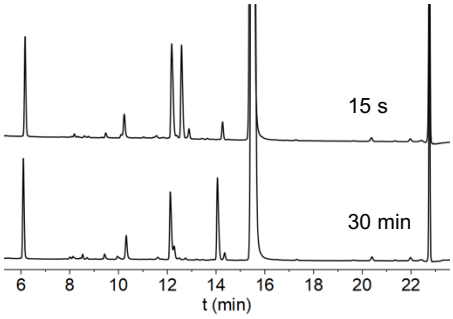
t = 5.6 min (L-Tyr), 8.4 min (CRAFS), 10.3 min (BtH), 11.4 min (LYRAACRAFS), 22.8 min (4-MPOH disulphide).

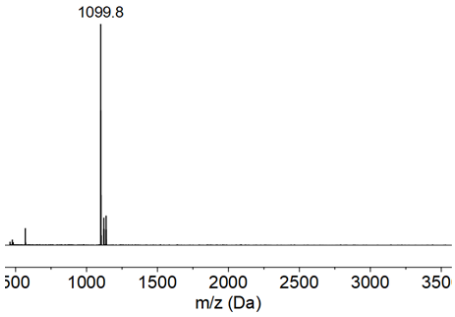
Calc. $[M+H]^+$: 1157.4, found: 1157.6.

Figure S42. LYRAACRAFS. Analytical HPLC of the ligation at time 15 s, 1 h. MALDI-TOF (right) of the ligation product.

LYRAGCLAFS





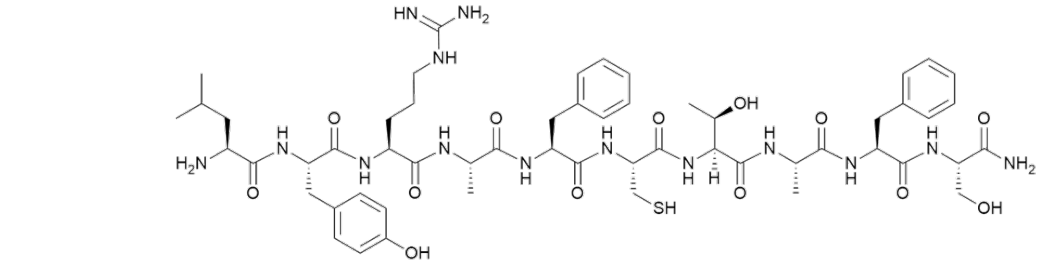


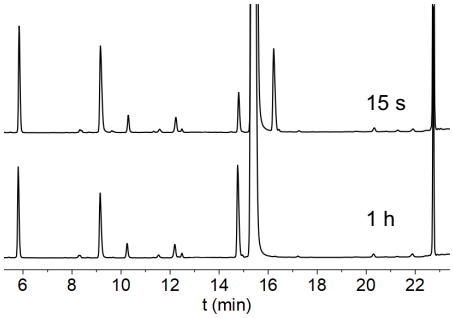
t = 5.7 min (L-Tyr), 10.4 min (BtH), 12.1 min (CLAFS), 12.6 min (LYRAG-(4-MPOH)), 14.0 min (LYRAGCLAFS), 22.7 min (4-MPOH disulphide).

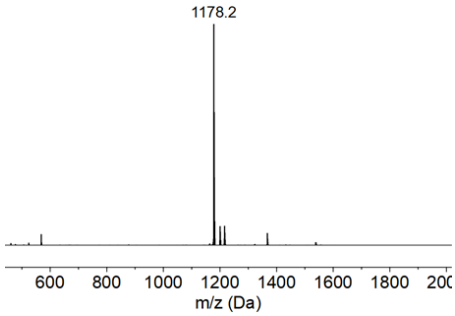
Calc. $[M+H]^+$: 1100.6, found: 1099.8.

Figure S43. LYRAGCLAFS. Analytical HPLC trace of the ligation at time 15 s, 30 min. MALDI-TOF (right) of the ligation product.

LYRAFCTAFS (32)





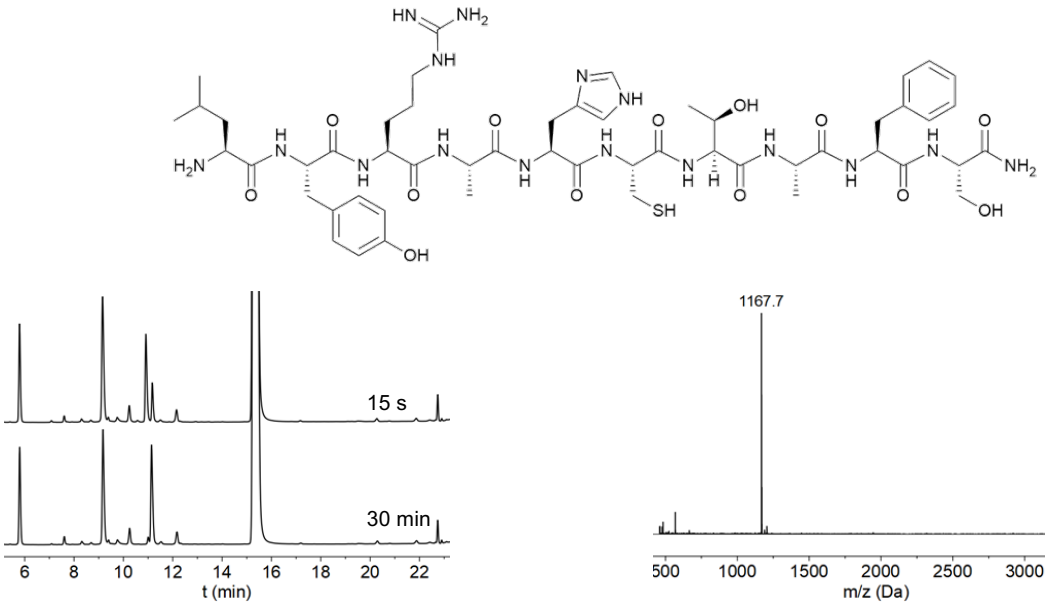


t = 5.9 min (L-Tyr), 9.2 min (CTAFS), 10.3 min (BtH), 14.7 min (LYRAFCTAFS), 16.4 min (LYRAF-(4-MPOH)), 22.7 min (4-MPOH disulphide).

Calc. $[M+H]^+$: 1178.4, found: 1178.2.

Figure S44. LYRAFCTAFS. Analytical HPLC trace of the ligation at time 15 s, 1 h. MALDI-TOF (right) of the ligation product.

LYRAHCTAFS

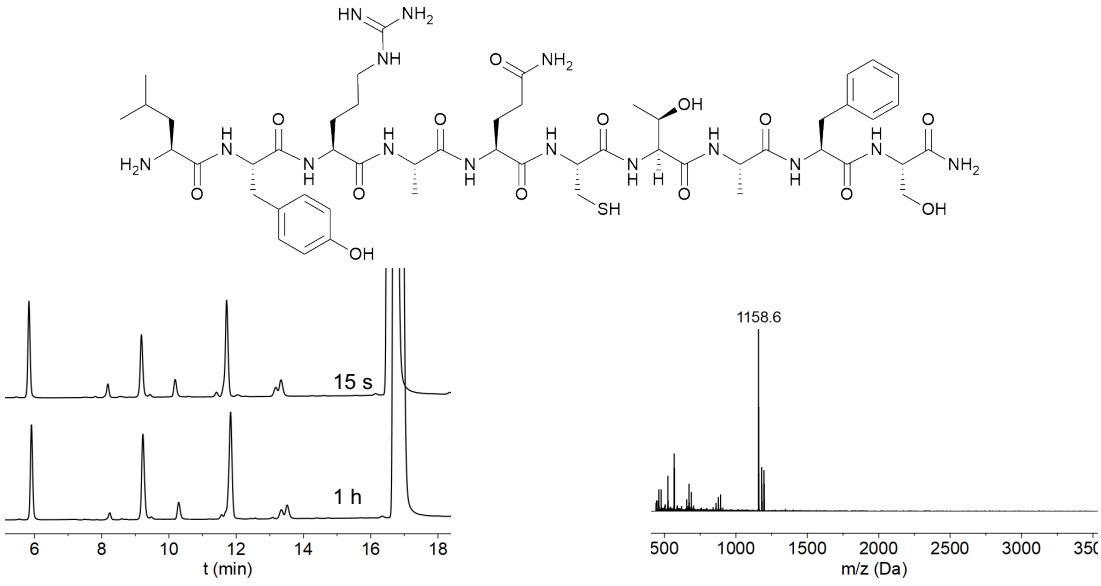


t = 5.8 min (L-Tyr), 9.2 min (CTAFS), 10.3 min (BtH), 10.9 min (LYRAH-(4-MPOH)), 11.1 min (LYRAHCTAFS).

Calc. $[M+H]^+$: 1168.4, found: 1167.7.

Figure S45. LYRAHCTAFS. Analytical HPLC trace of the ligation at time 15 s, 30 min. MALDI-TOF (right) of the ligation product.

LYRAQCTAFS

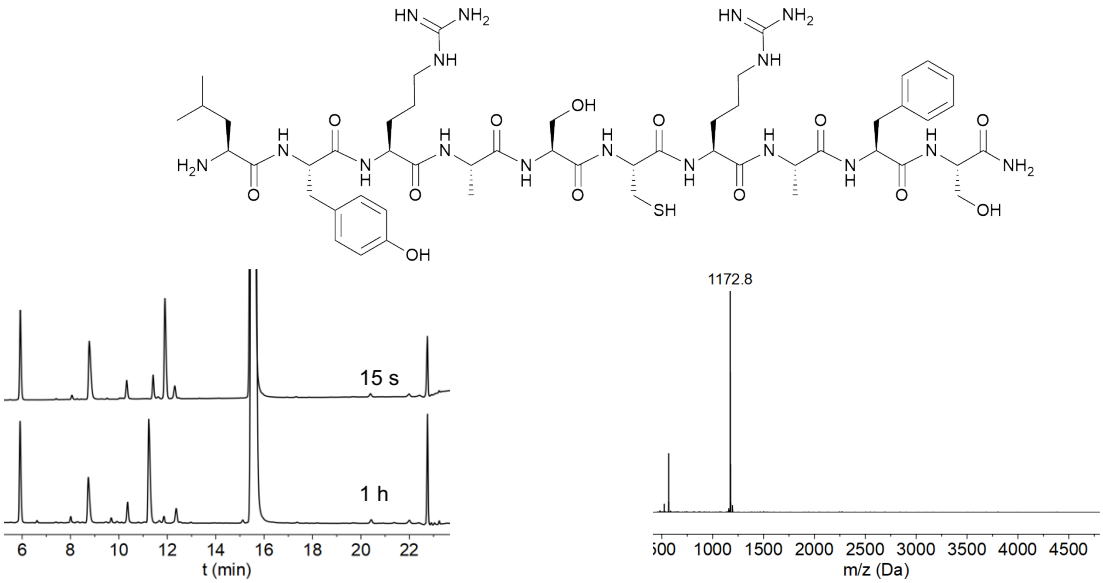


t = 5.9 min (L-Tyr), 9.2 min (CTAFS), 10.3 min (BtH), 11.7 min (LYRAQ-(4-MPOH)), 11.8 min (LYRAQCTAFS).

Calc. $[M+H]^+$: 1158.4, found: 1158.6.

Figure S46. LYRAQCTAFS. Analytical HPLC trace of the ligation at time 15 s, 1 h. MALDI-TOF (right) of the ligation product.

LYRASCRAFS

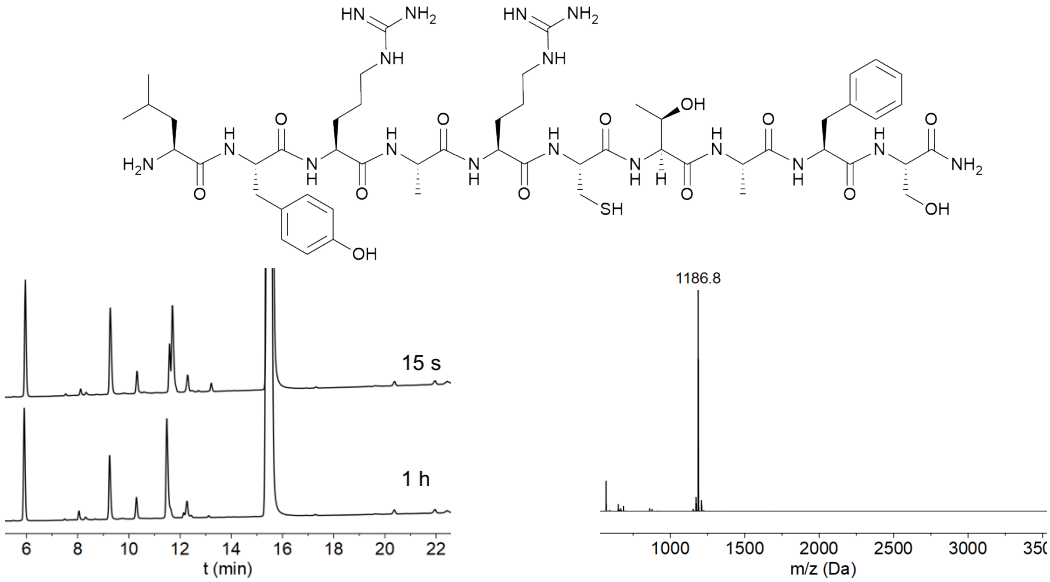


t = 5.9 min (L-Tyr), 8.7 min (CRAFS), 10.4 min (BtH), 11.2 min (LYRASCRAFS), 11.8 min (LYRAS-(4-MPOH)), 22.8 min (4-MPOH disulphide).

Calc. $[M+H]^+$: 1173.4, found: 1172.8.

Figure S47. LYRASCTAFS. Analytical HPLC trace of the ligation at time 15 s, 1 h. MALDI-TOF (right) of the ligation product.

LYRARCTAFS

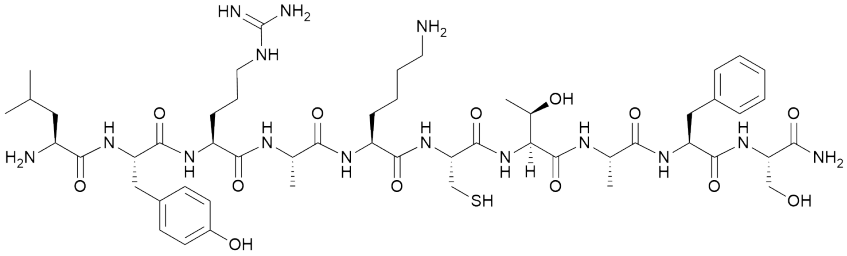


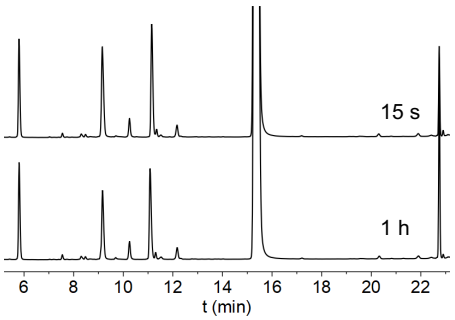
t = 5.9 min (L-Tyr), 8.0 min (LYRAR-OH), 9.2 min (CTAFS), 10.3 min (BtH), 11.2 min (LYRARCTAFS), 11.6 min (LYRAR-(4-MPOH)).

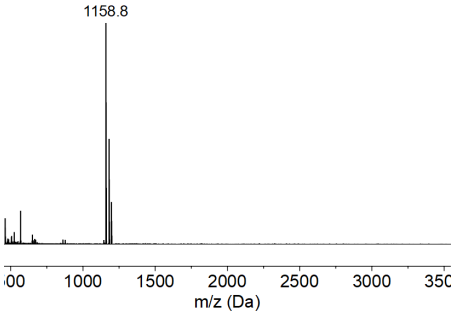
Calc. $[M+H]^+$: 1187.6, found: 1186.8.

Figure S48. LYRARCTAFS. Analytical HPLC trace of the ligation at time 15 s, 1 h. MALDI-TOF (right) of the ligation product.

LYRAKCTAFS





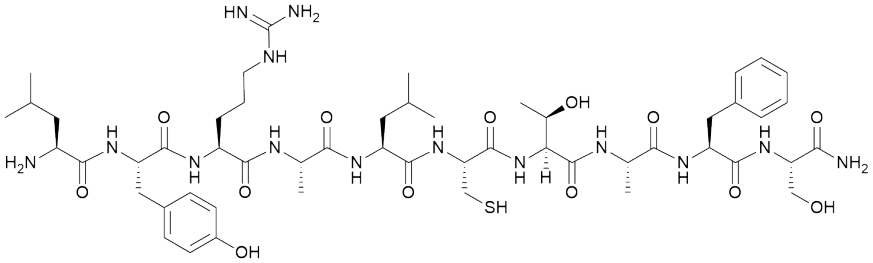


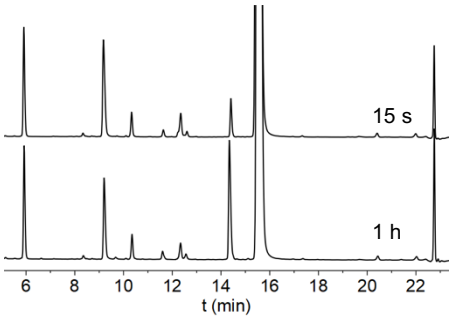
t = 5.8 min (L-Tyr), 9.2 min (CTAFS), 10.3 min (BtH), 11.1 min (LYRAKCTAFS), 11.2 min (LYRAK-(4-MPOH)), 22.7 min (4-MPOH disulphide).

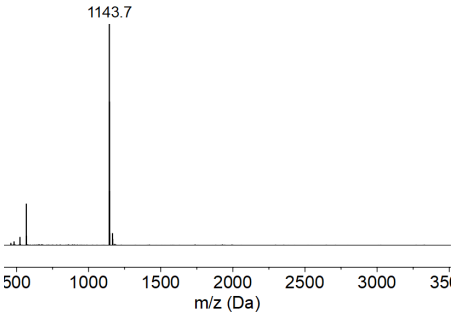
Calc. $[M+H]^+$: 1159.6 found: 1158.8.

Figure S49. LYRAKCTAFS. Analytical HPLC trace at 220 nm of the ligation at time 15 s, 1 h. MALDI-TOF (right) of the ligation product.

LYRALCTAFS





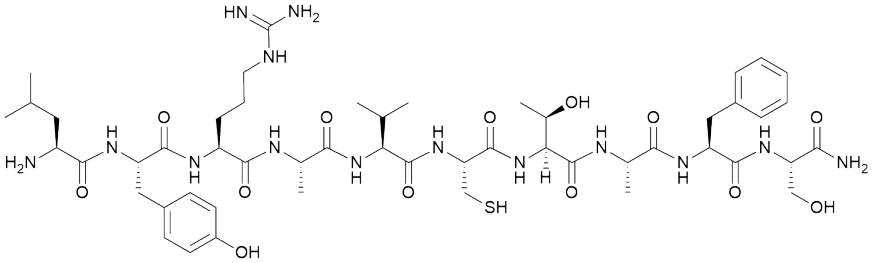


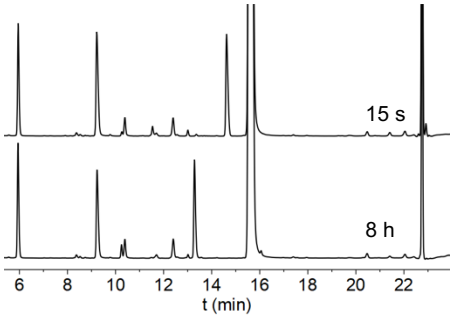
t = 5.9 min (L-Tyr), 9.2 min (CTAFS), 10.4 min (BtH), 14.3 min (LYRALCTAFS), 22.8 min (4-MPOH disulphide).

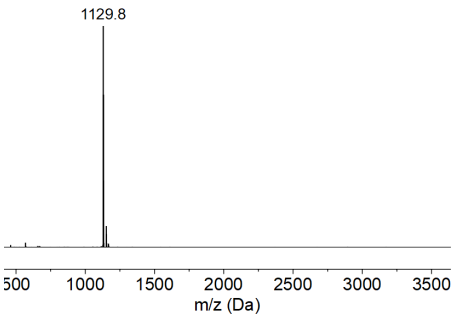
Calc. $[M+H]^+$: 1144.6, found: 1143.7.

Figure S50. LYRALCTAFS Analytical HPLC trace of the ligation at time 15 s, 1 h. MALDI-TOF (right) of the ligation product.

LYRAVCTAFS





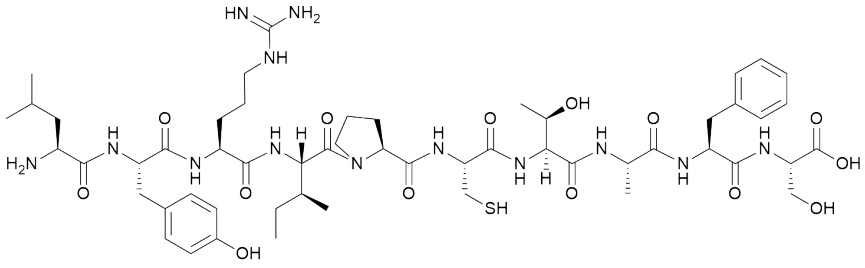


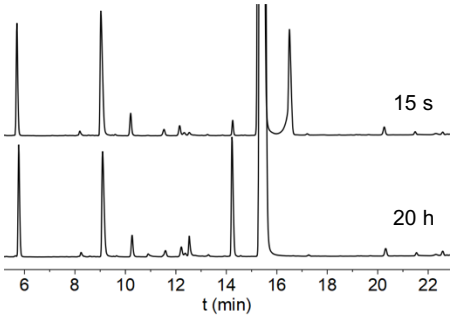
t = 5.9 min (L-Tyr), 9.2 min (CTAFS), 10.4 min (BtH), 13.3 min (LYRAVCTAFS), 14.7 min (LYRAV-(4-MPOH)), 22.8 min (4-MPOH disulphide).

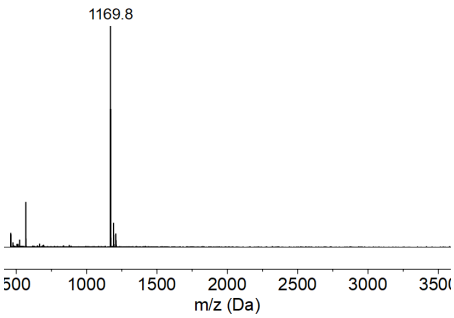
Calc. $[M+H]^+$: 1130.6, found: 1129.8.

Figure S51. LYRAVCTAFS. Analytical HPLC trace of the ligation at time 15 s, 8 h. MALDI-TOF (right) of the ligation product.

LYRIPCTAFS







t = 5.7 min (L-Tyr), 8.9 min (CTAFS), 10.2 min (BtH), 12.1 min (LYRIP-OH), 14.0 min (LYRIPCTAFS), 16.3 min (LYRIP-(4-MPOH)).

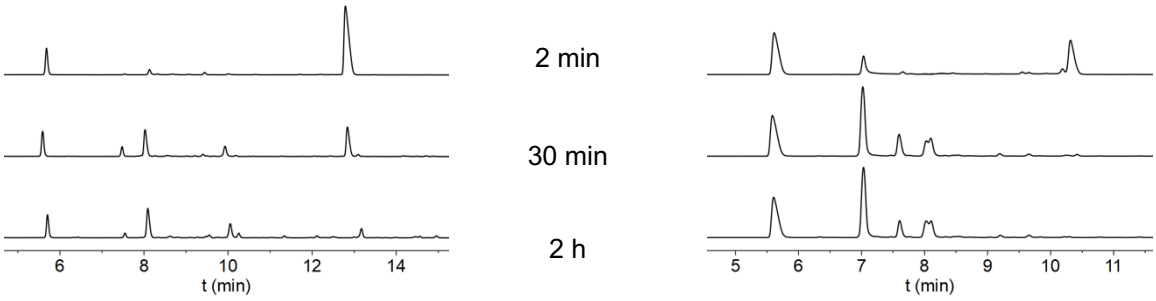
Calc. $[M+H]^+$: 1170.6, found: 1169.8.

Figure S52. LYRIPCTAFS. Analytical HPLC trace of the ligation at time 15 s, 20 h. MALDI-TOF (right) of the ligation product.

8. Stability of LYRAG-Bt and LYRAG-Bt-CONH-G

In two separated vials, LYRAG-Dbz (1 mg, $1.5x10^{-3}$ mmol) and LYRAG-Dbz-CONH-G (1 mg, $1.3x10^{-3}$ mmol) were dissolved in degassed buffer (6 M Gdm.HCl, 0.2 M NaPhos, 0.19 mL, pH = 7.0) containing L-Tyr (2 mM) at room temperature. Next, a solution of $NaNO_2$ in buffer (10 µL from a 0.5 M stock, $5x10^{-3}$ mmol) was added and the reactions monitored by direct injection in HPLC.

To calculate the ratio of remaining LYRAG-Bt and LYRAG-Bt-CONH-G, peak areas (220 nm) at the selected time points were normalized to the area at 2 min (t_0, 100%). Note that at time = 2 min (first HPLC run), all starting LYRAG-Dbz and LYRAG-Dbz-CONH-G was converted into LYRAG-Bt and LYRAG-Bt-CONH-G.



Column A. t = 5.7 min (L-Tyr), 8.1 min (LYRAG-OH), 10.2 min (BtH), 12.8 min (LYRAG-Bt).

Column A. t = 5.7 min (L-Tyr), 7.0 min (BtH-CONH-G), 8.1 min (LYRAG-OH), 10.3 min (LYRAG-Bt-CONH-G).

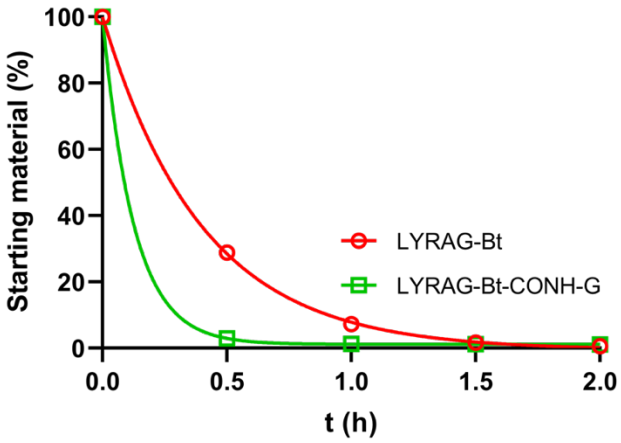


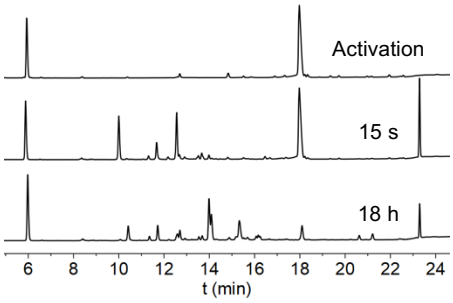
Figure S53. HPLC monitoring of the stability of LYRAG-Bt (left) and LYRAG-Bt-CONH-G (right). Bottom: representation of the ratio of remaining LYRAG-Bt (red) and LYRAG-Bt-CONH-G (green) as a function of time.

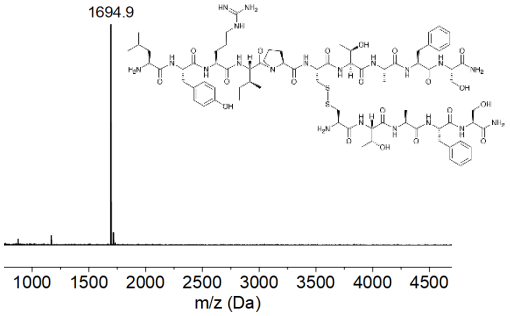
9. Bt-direct ligation between LYRIP-Dbz and CTAFS (27)

LYRIP-Dbz (1 mg, $1.3x10^{-3}$ mmol) was dissolved in degassed buffer (6 M Gdm.HCl, 0.2 M NaPhos, 0.19 mL, pH = 3.0) containing L-Tyr (6 mM). The solution was cooled down at ~0 °C in an ice bath and $NaNO_2$ (10 µL from a 0.5 M stock, $5x10^{-3}$ mmol) was added. The reaction was left in the ice bath for 30 min.

Following LYRIP-Bt formation, a solution of buffer (6 M Gdm.HCl, 0.2 M NaPhos, 0.4 mL, pH = 7.0) containing **27** (1.5 mg, $2.8x10^{-3}$ mmol) and ^{t}BuSH (10 µL, $8.9x10^{-2}$ mmol) was added. The resulting mixture (final pH = 6.2) was stirred at room temperature. The ligation was monitored by HPLC by taking aliquots that were quenched with $HCl_{(aq)}$ (0.5 M) at the indicated times. Product identification was carried out by MALDI-TOF analysis of the products corresponding to the different HPLC peaks.

The ligation product LYRIPCTAFS was found at a retention time = 14.0 min as the oxidized disulphide LYRIP$(CTAFS)_2$.





Column A. t = 6.0 (L-Tyr), 9.7 min (**27**), 10.4 min (BtH), 14.0 min (LYRIP$(CTAFS)_2$), 17.9 min (LYRIP-Bt), 23.3 min (^{t}BuS)$_2$.

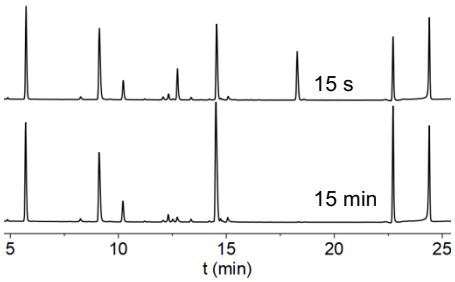
Calc. LYRIP$(CTAFS)_2$ $[M+H]^+$: 1695.0, found: 1694.9.

Figure S54. LYRIPCTAFS. Analytical HPLC trace of the ligation at time 15 s, 18 h. MALDI-TOF (right) of the ligation product.

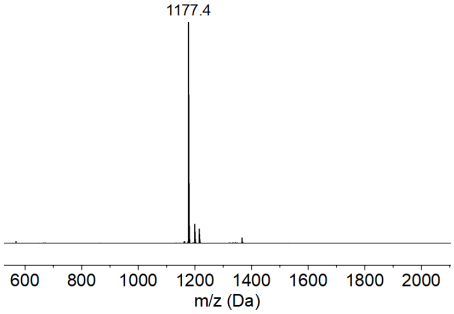
10. Chemical ligation mediated by selenoesters: LYRAF-COSePh.

LYRAF-Dbz (**29**, 1 mg, $1.3x10^{-3}$ mmol) was dissolved in degassed buffer (6 M Gdm.HCl, 0.2 M NaPhos, 0.19 mL, pH = 3.0) containing L-Tyr (6 mM) and cooled down in an ice-salt bath (~ –15 °C). A solution of $NaNO_2$ (10 µL from a 0.5 M stock, $5x10^{-3}$ mmol) was added. The reaction was left in the ice-salt bath for 30 min.

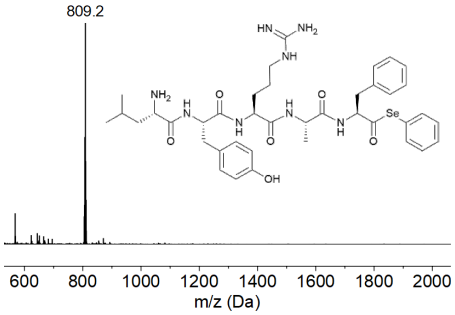
In parallel, a mixture of diphenyl diselenide (DPDS, 50 mM) and TCEP.HCl (100 mM) in degassed buffer (6 M Gdm.HCl, 0.2 M NaPhos, 0.4 mL, pH = 8.5) was sonicated until complete reduction of DPDS to benzeneselenol, i.e. homogenous solution. Next, the pH was adjusted to 6.7 and this mixture was added to peptide **27** (1.5 mg, $2.8x10^{-3}$ mmol). The resulting solution was mixed with the LYRAF-Bt peptide (final pH = 6.2) and stirred at room temperature. HPLC monitoring was carried out by taking aliquots at the indicated time that were quenched with the same volume of $HCl_{(aq)}$ (0.5 M). Product identification was carried out by MALDI-TOF analysis of the products corresponding to the different HPLC peaks.



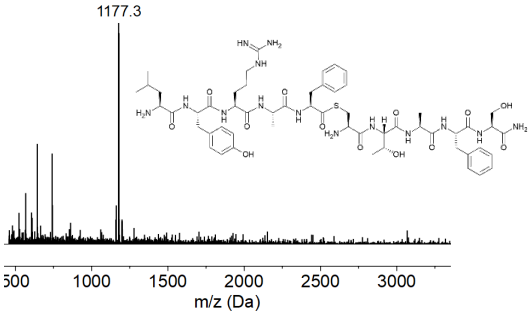
Column A. t = 5.7 min (L-Tyr), 9.1 min (CTAFS), 10.2 min (BtH), 12.7 min (**31**), 14.5 min (**32**), 18.3 min (**30**), 22.7 min (PhSeH), 24.4 min (DPDS).



Calc. **32** $[M+H]^+$: 1178.4, found: 1177.4.



Calc. **30** $[M+H]^+$: 808.9, found: 809.2.



Calc. **31** $[M+H]^+$: 1178.4, found: 1177.3.

Figure S55. LYRAFCTAFS. Analytical HPLC trace of the ligation at time 15 s, 15 min. MALDI-TOF of the ligation product (**32**), LYRAF-COSePh (**30**) and LYRAF(S)CTAFS (**31**).

11. Synthesis of GR DBD

11.1. Synthesis of F1-(*p*CN-Phoc)Dbz: KLCLVCSDEASGCHYGVLT-(*p*CN-Phoc)Dbz

Synthesis was carried out on AM-ChemMatrix resin (0.56 mmol/g, 1 g). Following the incorporation of the PAL linker, the PAL-AM-resin was stirred overnight in a DMF solution containing 2-fluoronitrobenzene (1 mL, 9.5 mmol) and DIEA (0.5 mL, 2.9 mmol). Next, the resin was transferred to a falcon tube (50 mL) and vortexed overnight in an orbital shaker (10 rpm) in the presence of an aqueous solution containing $Na_2S_2O_4/K_2CO_3$ (1:1, 0.5 M, 40 mL). Following the reduction of the nitro group, Fmoc-Thr(^{t}Bu)-OH was coupled using a 4-fold excess (2.0 mmol) preactivated 20 s with HATU (0.760 g, 2.0 mmol) and DIEA (0.52 mL, 3.0 mmol). Chain elongation was carried out using the Fmoc-amino acids (2.5 mmol) in the presence of DIC (0.425 mL, 2.7 mmol), 6-Cl-HOBt (0.424 g, 2.5 mmol) and DIEA (0.22 mL, 1.3 mmol). Coupling times were 45 min. When necessary, double couplings were performed using HATU activation. Fmoc removal was performed with a solution of piperidine in DMF (20%, 2x5 min). The last amino acid was introduced as Boc-Lys(Boc)-OH.

Following peptide assembly, the dry resin (0.5 g) was swollen in DCM. Next, a solution of *p*-cyanophenyl chloroformate (210 mg, 1.2 mmol) and 2,4,6-trimethylpyridine (0.045 mL, 0.34 mmol) in DCM (10 mL) was added to the resin and the resulting mixture stirred at room temperature for 90 min. Next, the resin was filtered off and washed with DCM, DMF and finally DCM, dried under vacuum and cleaved using TFA/H_2O/TIS/thioanisole (88:2:5:5, 30 mL) for 2 h 30 min. After standard workup (section 2), the solid was lyophilized affording 137 mg of crude peptide (70% recovery), and purified by semi-preparative HPLC (69 mg of F1-(*p*CN-Phoc)Dbz, 50% based on the recovery peptide).

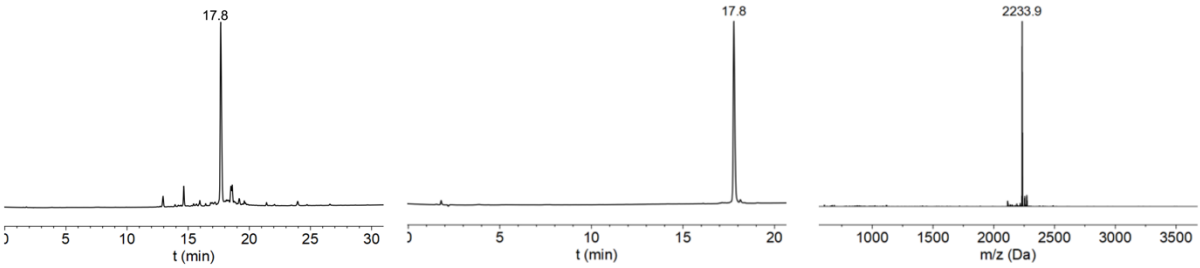
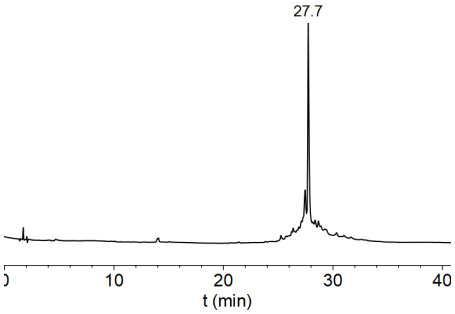


Figure S56. F1-(*p*CN-Phoc)Dbz. HPLC trace of the crude synthesis of F1-(*p*CN-Phoc)Dbz (left, column A: 0 to 70% buffer B in 30 min) and the purified peptide (middle, column A). MALDI-TOF of a purified sample of F1-(*p*CN-Phoc)Dbz (right). Calc. $[M+H]^+$: 2234.6, found = 2233.9.

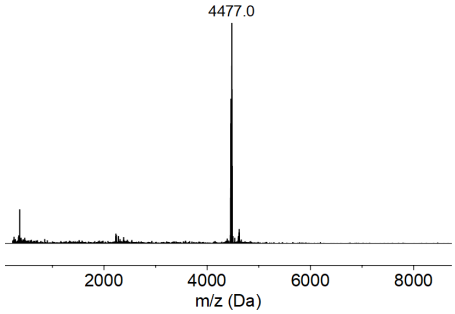
11.2. Synthesis of F2(Acm)-Dbz: C(Acm)GSCKVFFKRAVEGQHNYLCAGRNDCIIDKIRRKNCPA-Dbz

Synthesis was carried out in a AM-ChemMatrix resin (0.56 mmol/g, 1 g), following a similar scheme to F1-(*p*CN-Phoc)Dbz. Different to the previous synthesis, after the introduction of the first Ala, in each coupling cycle was used the Fmoc-amino acid (3.0 mmol), DIC (0.5 mL, 3.2 mmol) and DIEA (0.26 mL, 1.5 mmol). The last amino acid was introduced as Boc-Cys(Acm)-OH.

Following chain elongation, the dry resin (1.9 g split in two separated vessels containing 1 and 0.9 g, respectively) was cleaved using TFA/H_2O/TIS/thioanisole (88:2:5:5, 40 mL for each cleavage) for 1 h 30 min. After standard workup (section 2), the solid was lyophilized affording 511 mg of crude peptide (62% recovery). The crude peptide F2(Acm)-Dbz was used in the next step without further purification.



Column A: 0 to 40% buffer B in 40 min. t = 27.7 min (F2(Acm)-Dbz).



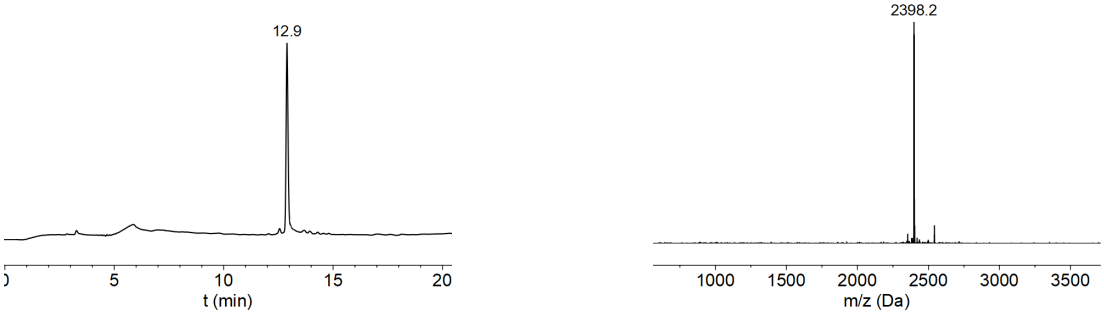
Calc. $[M+H]^+$: 4777.3, found: 4477.0.

Figure S57. F2(Acm)-Dbz. HPLC trace of the crude synthesis of F2(Acm)-Dbz and MALDI-TOF of the compound corresponding to the peak at 27.7 min (right).

11.3. Synthesis of F3: CRYRKCLQAGMNLEARKTKK^{-OH}

Synthesis was carried out in a 2-Cl-trityl resin (1.6 mmol/g, 1.5 g). The resin was first rinsed with DCM, filtered off and a solution of Fmoc-(Lys)-OH (234 mg, 0.5 mmol) and DIEA (0.69 mL, 4 mmol) in DCM (6 mL) was added to the resin. The resulting suspension was gently stirred at room temperature for 4 h. Then, the resin was filtered off, washed with DCM and a solution of MeOH (5%) and DIEA (1%) in DCM. Chain elongation was carried out using the Fmoc-amino acids (2.0 mmol) preactivated for 30 s with HBTU (0.76 g, 2.0 mmol) and DIEA (0.52 mL, 3 mmol). Coupling times were 45 min. When necessary, double couplings were performed using HATU activation. Fmoc removal was performed with a solution of piperidine in DMF (20%, 2x5 min). The last amino acid was introduced as Boc-Cys(Trt)-OH.

Following peptide assembly, the dry resin was cleaved using a solution of TFA (10%), H_2O (1%) and TIS (1%) in DCM. The protected peptide was collected in a round-bottom flask and the DCM evaporated under reduced pressure in a rotary evaporator. Next, the side chain protecting groups were removed using the standard cleavage cocktail (TFA/TIS/H_2O). After standard workup (section 2), the solid was lyophilized and purified by semi-preparative HPLC (225 mg of purified F3).

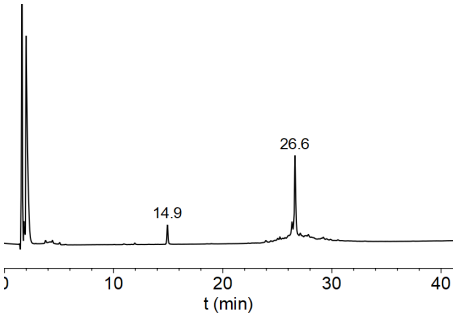


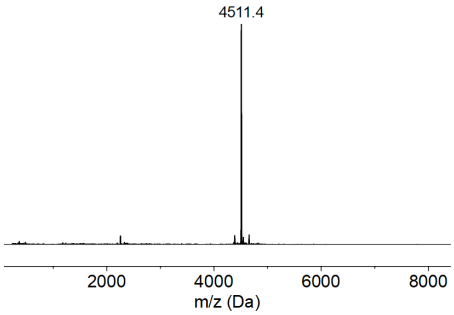
Column A. Calc. $[M+H]^+$: 2398.9, found: 2398.2.

Figure S58. F3. Analytical HPLC trace of the purified F3. MALDI-TOF of the purified F3 peptide (right).

11.4. Synthesis of F2(Acm)-MESH: C(Acm) GSCKVFFKRAVEGQHNYLCAGRNDCIIDKIRRKNCPA-MESH

The crude peptide F2(Acm)-Dbz (60 mg, ~$1.3x10^{-2}$ mmol) was dissolved in degassed buffer (6 M Gdm·HCl, 0.2 M NaPhos, 2 mL, pH = 3), and the solution cooled down in an ice-salt bath at –15ºC. $NaNO_2$ (100 µL of a freshly prepared 1 M solution, 0.1 mmol) was added and the reaction left in the ice-salt bath for 30 min. Following Bt formation, a solution of degassed buffer (6 M Gdm.HCl, 0.2 M NaPhos, 5 mL, pH = 7) containing MESNa (82 mg, 0.5 mmol) and TCEP.HCl (28.6 mg, 0.1 mmol) was added and the pH adjusted to 6.7. The reaction was left at room temperature for 30 min, then acidified until pH = 5 using $HCl_{(aq)}$ (37%) and finally purified by semi-preparative HPLC. It was recovered 22.6 mg after lyophilization (39% based on the F2(Acm)-Dbz crude).





Column A: 0 to 40% buffer B in 40 min. t = 14.9 min (BtH), t = 26.6 min (F2(Acm)-MESH).

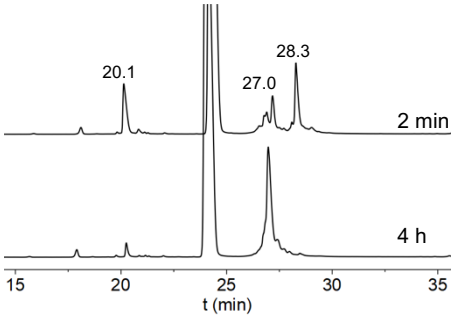
Calc. $[M+H]^+$: 4511.3, found: 4511.4.

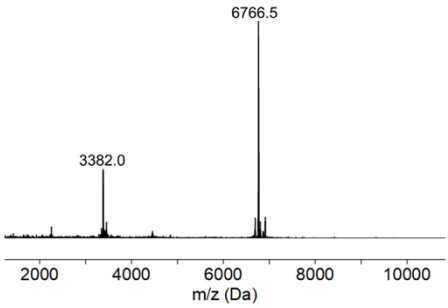
Figure S59. F2(Acm)-MESH. HPLC trace of the crude synthesis of F2(Acm)-MESH and MALDI-TOF of the compound corresponding to the peak at 26.6 min (right).

11.5. Ligation between F2(Acm)-MESH and F3: F2(Acm)-F3 C(Acm)GSCKVFFKRAVEGQHNYLCAGRNDCIIDKIRRKNCPACRYRKCLQAGM NLEARKTKK^{-OH}

F2(Acm)-MESH (18.3 mg, $4x10^{-3}$ mmol) and F3 (13.0 mg, $5.4x10^{-3}$ mmol) were dissolved in in degassed ligation buffer (6 M Gdm.HCl, 0.2 M NaPhos, 1 mL, pH = 7) containing 4-MPOH (200 mM) and TCEP.HCl (50 mM). The ligation was monitored by direct injection in HPLC at the indicated times.

Following completion of the reaction, the pH of the solution was adjusted to 5.5 and 4-MPOH extracted using Et_2O (3x0.5 mL). The product was isolated by semi-preparative HPLC and lyophilized, affording 18.0 mg of purified F2(Acm)-F3 (67%).





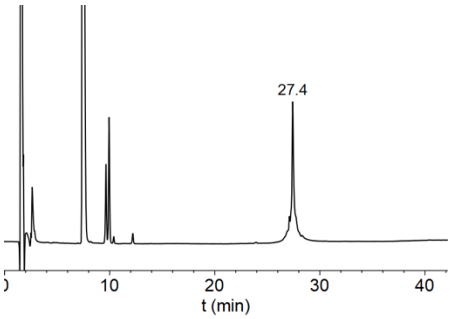
Column A: 0 to 40% buffer B in 40 min. t = 20.1 min (F3), 27.0 min (F2(Acm)-F3), 28.3 min (F2(Acm)-MESH).

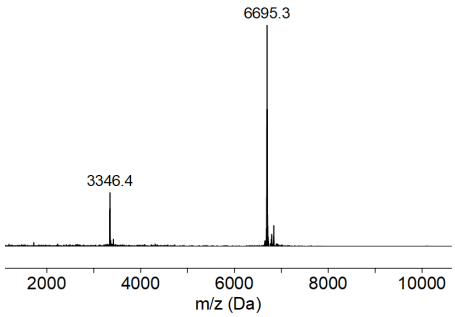
Calc. $[M+H]^{+}$: 6767.1, $[M+2H]^{2+}/2$: 3383.6. Found: 6766.5, 3382.0.

Figure S60. F2(Acm)-F3. Analytical HPLC trace of the ligation at time 2 min, 4 h. MALDI-TOF of the compound corresponding to the peak at 27.0 min (right).

11.6. Acm removal from F2(Acm)-F3: F2-F3 CGSCKVFFKRAVEGQHNYLCAGRNDCIIDKIRRKNCPACRYRKCLQAGMNLEARKTKK^{-OH}

F2(Acm)-F3 (11.4 mg, $1.7x10^{-3}$ mmol) was dissolved in a degassed H_2O/AcOH mixture (1:1, 1 mL) containing AgOTf (12.8 mg, $5x10^{-2}$ mmol) and stirred at room temperature for 6 h. The reaction was quenched by the addition of a DTT solution (38.5 mg, 0.25 mmol) in degassed buffer (6 M Gdm.HCl, 0.2 M NaPhos, 1 mL) adjusted to a final pH = 7. After stirring for 10 min, the suspension was spun out and the supernatant collected. The pellet was washed again with DTT solution (3x1 mL) and the liquid fractions directly purified by semi-preparative HPLC. After lyophilization, it was obtained 8.1 mg of purified F2-F3 (71%).





Column A: 0 to 40% buffer B in 40 min.

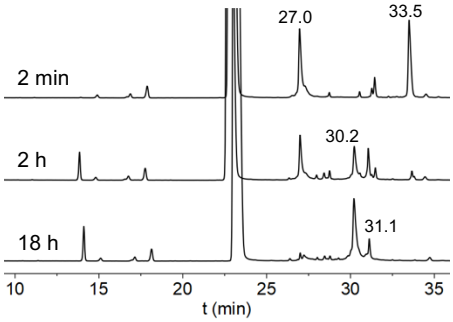
Calc. $[M+H]^{+}$: 6696.0, $[M+2H]^{2+}/2$: 3348.0. Found: 6695.3, 3346.4.

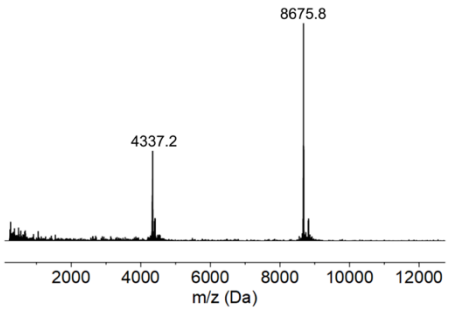
Figure S61. F2-F3. F2-F3. Analytical HPLC trace of the crude Acm removal after DTT quenching. MALDI-TOF of the compound corresponding to the peak at 27.4 min (right).

11.7. Ligation of fragments F1 and F2-F3: F1-F2-F3 KLCLVCSDEASGCHYGVLTCGSCKVFFKRAVEGQHNYLCAGRNDCIIDKIRRK NCPACRYRKCLQAGMNLEARKTKK^{-OH}

F2-F3 (7.0 mg, 1.0x10^{-3} mmol) and F1-(*p*CN-Phoc)Dbz (2.6 mg, 1.2x10^{-3} mmol) were dissolved in degassed ligation buffer (6 M Gdm.HCl, 0.2 M NaPhos, 200 mM 4-MPOH, 50 mM TCEP.HCl, 0.7 mL, pH = 7) and stirred until completion (overnight) at room temperature.

The reaction was quenched by acidification until pH = 2, and the 4-MPOH extracted using Et_2O (3x0.5 mL). The aqueous solution containing the peptide was purified by semi-preparative HPLC and the product lyophilized, giving 4.0 mg of F1-F2-F3 (46%).





Column A: 0 to 40% buffer B in 40 min. t = 14.0 min (Nbz), 18.0 min (*p*-cyanophenol), 27.0 min (F2-F3), 30.2 min (F1-F2-F3), 31.1 min (F1-(4-MPOH)), 31.5 min (F1-Nbz), 33.5 min (F1-(*p*OH-phenylthiocarbonyl)Dbz.

Calc. $[M+H]^+$: 8676.3, $[M+2H]^{2+}/2$: 4338.1. Found: 8675.8, 4337.2

Figure S62. F1-F2-F3. Analytical HPLC of the ligation at time 2 min, 2 h, 6 h. MALDI-TOF of the compound corresponding to the peak at 30.2 min (right).

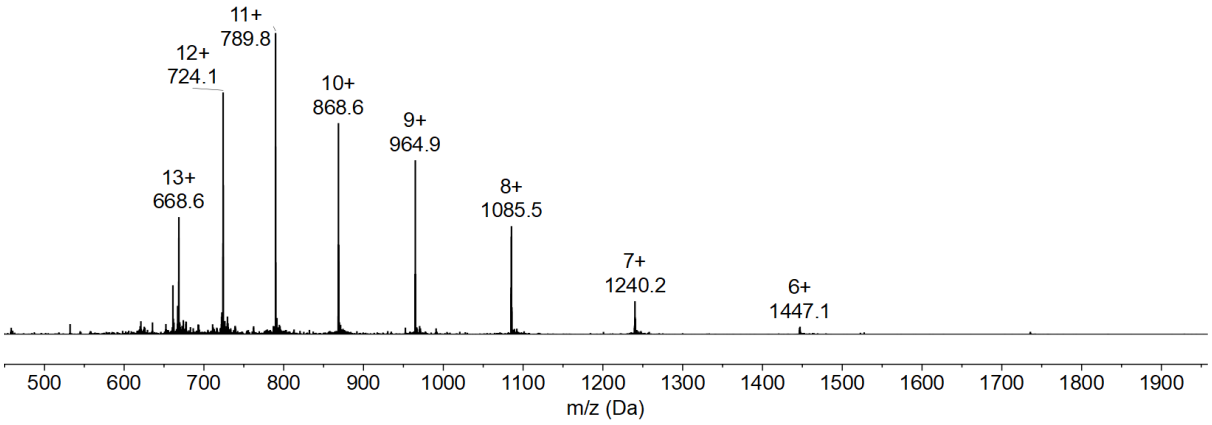


Figure S63. ESIMS of F1-F2-F3 (linear GR DBD, Figure 6B manuscript).

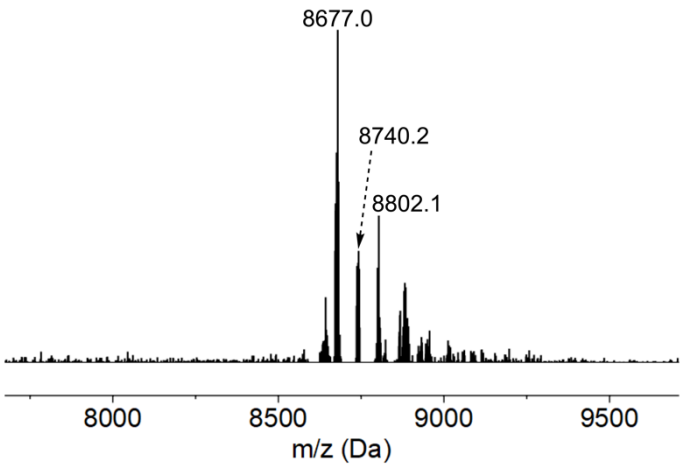


Figure S64. MALDI-TOF of GR DBD (10 μM) in the presence of $ZnCl_2$ (100 μM) in NH_4OAc buffer (10 mM, pH = 6.0). Calc. $[M+H]^+$: 8676.3, [M+Zn-3H]: 8737.6, [M+2Zn-7H]: 8799.0. Found: 8677.0, 8740.2, 8802.1.

11.8. Electrophoretic mobility shift assays (EMSA)

Electrophoretic mobility shift assays were carried out in polyacrylamide gels polymerized using a solution of Acrylamide/Bisacrylamide 37.5:1 (40% w/v solution, Merck) in a Tris-Borate (1:1, 45 mM) buffer at pH = 8.3. The gel was prepared containing a biphasic composition: top (stacking) at 4%, and bottom (resolving) at 10%. Gels were run at ~12 °C, the first 10 min at 100 V and for another 30 min at 140 V. Staining was performed using SyBR Gold (1:10000 dilution, Thermo Fischer) in a solution of Tris-Borate (90 mM) at pH 8.3 for 10 min. Fluorescence images were registered in a Kodak Gel Logic 1500 imaging system run with Kodak Molecular Imaging Software, and the pictures processed with the GIMP software (https://www.gimp.org/).

For EMSA and circular dichroism experiments, protein concentration was determined at 280 nm by the method of Moffatt et al.[4] using L-Tyr as calibration standard.

Increasing concentrations of linear F1-F2-F3 (0, 0.6, 0.8, 1.0, 1.4, 1.8, 2.3 μM) were incubated in the presence of the dsGRE (5'-CGC CAG AAC AAA ATG TTC TGG CG-3', 100 nM) at 4 °C for 2 h in a buffer containing: Tris (18 mM, pH = 7.6), KCl (90 mM), $MgCl_2$ (1.8 mM), DTT (0.2 mM), $ZnCl_2$ (0.04 mM) and NP-40 (10% v/v). Samples were diluted with glycerol (10% v/v) before loading in the gel. The composition of the running buffer was Tris-Borate (1:1, 45 mM, pH = 8.3).

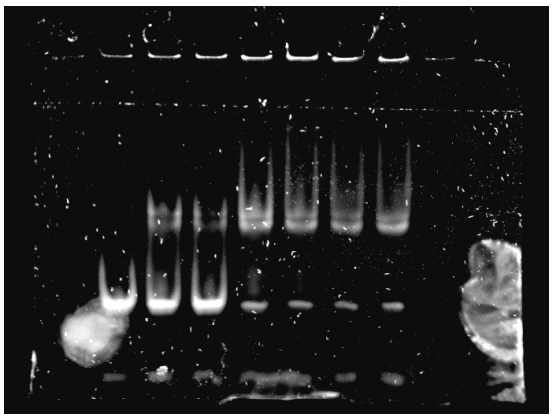


Figure S65. Raw picture of the EMSA experiment.

11.9. Circular Dichroism of GR DBD

Linear F1-F2-F3 (35 μM) in buffer (Tris (18 mM), KCl (90 mM), $MgCl_2$ (1.8 mM), DTT (0.2 mM), pH = 7.6) was titrated at 22 °C in the presence of increasing concentrations of $ZnCl_2$ (0, 5, 10, 20, 40 μM), with preincubation time of 2 min after addition of the corresponding concentration of $ZnCl_2$ (Figure 6C manuscript).

Following the addition of the last aliquot of $ZnCl_2$ (40 μM), dsGRE (5 μM) was added and incubated in the presence of [GR DBD] = 35 μM.

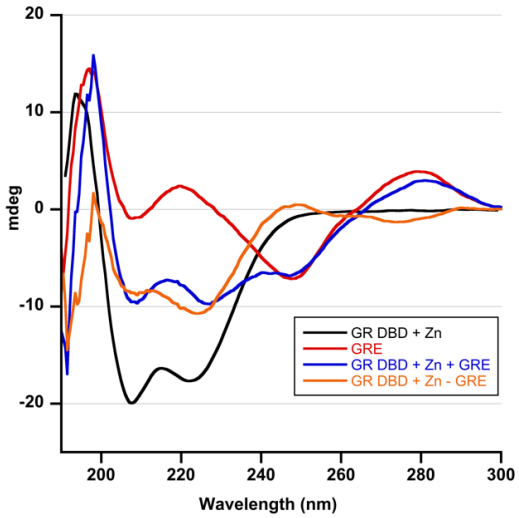


Figure S66. Circular dichroism of GR DBD in the presence of Zn and GRE. [GR DBD] = 35 μM, [$ZnCl_2$] = 40 μM, [dsGRE] = 5 μM.

References:

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