A straightforward method for automated Fmoc-based synthesis of bio-inspired peptide crypto-thioesters

Victor P. Terrier, Hélène Adihou, Mathieu Arnould, Agnès F. Delmas, Vincent Aucagne
Centre de Biophysique Moléculaire, CNRS UPR 4301, Rue Charles Sadron 45071 Orléans cedex 2, France.

Supporting Information

Table of contents:

1) General information S2
2) General procedures for solid phase peptide synthesis S3
3) Optimization of the benzyl group to maximize the N-acylation yield S4
   a- Synthesis of cysteinyln peptide resin 1 S4
   b- Synthesis of peptide resins 2a-d S5
   c- Study on the solid-supported N-acylation of 2a-d S9
4) Evaluation of the phenol pKa of an N-acyl-N-(Hnb)Cys compound S3 S12
5) Characterization of a model N-acyl-N-(Hnb)Cys compound S5 S14
   a- NMR of S5 S14
   b- Molar extinction coefficient of the Hnb group of S5 S24
6) Optimization of the cysteine thiol protecting group S24
   a- Synthesis and stability of (S-Trt) model peptide S7 S24
   b- Synthesis and stability of (S-StBu) model peptide 7 S28
7) Optimization of the spacer between the cysteine and the resin S30
   a- Direct attachment to Rink linker – amide hydrolysis co-product S30
   b- Direct attachment to PHB Tentagel resin (Wang type linker) S31
   c- Introduction of a Gly residue as a spacer S32
8) N-Acylation with the 20 different proteogenic amino acids S34
9) Kinetics studies of the NCL with model Ac-LYRAA(Hnb)C peptides S57
   a- Synthesis of model cysteinyln peptide 6 S57
   b- Influence of the nature of the cysteine C-terminus S59
   c- Study of the hydrolysis of the thioester precursor 9 and epimerization S62
   d- Influence of the buffer S64
   e- Influence of masking phenol or thiol groups S65
   f- Influence of the pH S71
10) Influence of the C-terminal aa: NCL with model Ac-LYRAX(Hnb)C peptides S73
    a- Synthesis of peptide Ac-LYRAS-(Hnb)C(SiBu)G-NH₂ 10 S73
    b- NCL with peptide Ac-LYRAS-(Hnb)C(SiBu)G-NH₂ 10 S75
    c- Synthesis of peptide Ac-LYRAV-(Hnb)C(SiBu)G-NH₂ 11 S76
    d- NCL with peptide Ac-LYRAV-(Hnb)C(SiBu)G-NH₂ 11 S78
    e- NCL with peptide 11 under optimized conditions S78
    f- Determination of apparent second order kinetic constants S79
11) Application to MT7 S83
1- General information

All reagents and solvents were used without further purification. Protected amino acids, Fmoc-Gly-Wang resin, Fmoc-Rink polystyrene resin, Rink linker, HBTU and HCTU were purchased from Merck Biosciences (Nottingham, UK). Aminomethyl Tentagel R resin and pre-loaded Fmoc-Cys(StBu)-PHB (Wang type) Tentagel resin were purchased from Rapp polymers (Tuebingen, Germany). Fmoc-K(Boc)-Mppa-OH was purchased from Polypeptide laboratories (Strasbour, France). Peptide synthesis grade DMF and HATU were obtained from Applied Biosystems (Courtaboeuf, France). Ultrapure water was obtained using a Milli-Q water system from Millipore (Molsheim, France). All other chemicals were from Sigma Aldrich (St-Quentin-Fallavier, France) and solvents from SDS-Carlo Erba (Val de Reuil, France).

1H and 13C NMR spectra were recorded on a Bruker AVANCE III 600 instrument, at a constant temperature of 25°C. Chemical shifts are reported in parts per million from low to high field and referenced to tetramethylsilane (TMS). Coupling constants (J) are reported in hertz (Hz). Standard abbreviations indicating multiplicity were used as follows: s = singlet, d = doublet, dd = doublet of doublets, t = triplet, m = multiplet, b= broad signal.

High resolution ESI-MS analyses were performed on a maXis ultra-high-resolution Q-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany), using the positive mode. MALDI-TOF analyses were performed on an Ultraflex instrument (Bruker Daltonics, Bremen, Germany) equipped with a 337-nm nitrogen laser and a gridless delayed extraction ion source. The sample was co-crystallized with a solution of α-cyano-4-hydroxy-cinnamic acid (HCCA) as a matrix. The reported m/z values correspond to the monoisotopic ions if not specified otherwise. Peptides incorporating an N-(2-hydroxy-5-nitrobenzyl) group displayed a typical MALDI fragmentation pattern, consisting in -16 Da and -34 Da minor peaks in addition to the [MH]+ peak.

HPLC analyses and semi-preparative purifications were carried out on a LaChrom Elite system equipped with a Hitachi L-2130 pump, a Hitachi L-2455 diode array detector and a Hitachi L-2200 autosampler. Nucleosil C18 (300 Å, 5 μm, 250 × 4.6 mm, 1 mL/min flow rate) or Chromolith HighResolution RP-18e (150 Å, 10 × 4.6 mm, 3 mL/min flow rate) columns were used for analysis and Nucleosil C18 (300 Å, 5 μm, 250 × 10 mm, 3 mL/min flow rate) for purification. Solvents A and B are 0.1 % TFA in
H₂O and 0.1 % TFA in MeCN, respectively. Each gradient was followed by a washing step (95% B/A over 0.5 min for the HR Chromolith; over 1 min for the Nucleosil C18 column) to identify eventual co-products not eluted during the gradient.

LC-MS analyses were carried out on a Ultimate® 3000 RS LIC HPLC system (Dionex, Germering, Germany), coupled with the maXis mass spectrometer and fitted with a Zorbax 300 SB-C18 RRHD (300 Å, 1.8 μm, 100 × 2.1 mm, 0.3 mL/min flow rate, 40°C) column. Solvents A and B were 0.1 % formic acid in H₂O and 0.08 % formic acid in MeCN, respectively. Gradient: 3 % B/A for 1 min, then 3 to 50 % B/A over 19 min.

2- General procedures for solid phase peptide synthesis

Fmoc-based solid phase peptide syntheses (SPPS) were carried out on either a Prelude synthesizer from Protein Technologies or a 433A synthesizer from Applied Biosystems. Microwave-assisted Fmoc-SPPS syntheses were carried out on an Initiator+ SP Wave synthesizer from Biotage.

The side-chain protecting groups used were Arg(Pbf), Asn(Trt), Asp(OtBu), Cys(Acm), Cys(Trt), Cys(SiBu), Glu(OtBu), Gln(Trt), His(Trt), Lys(Boc), Ser(tBu), Thr(tBu), Trp(Boc) and Tyr(tBu).

Syntheses starting from aminomethyl Tentagel R resin were performed on the Prelude synthesizer, on a 0.025 mmol per reactor scale. Protected amino acids (0.25 mmol, 10 equiv.) were coupled using HCTU (98 mg, 0.238 mmol, 9.5 equiv.) and iPr₂NEt (87 μL, 0.5 mmol, 20 equiv.) in NMP (3 mL) for 30 min. Capping of eventual unreacted amine groups was achieved by treatment with acetic anhydride (143 μL, 1.51 mmol, 60 equiv.), iPr₂NEt (68 μL, 0.39 mmol, 15.5 equiv.) and HOBt (6 mg, 0.044 mmol, 1.8 equiv.) in NMP (3 mL) for 7 min. Fmoc group was deprotected by three successive treatments with 20 % piperidine in NMP (3 mL) for 3 min. In the cases where the coupling yield of an amino acid on the Hnb device is low, a washing step of the peptide-resin using a solution of hydroxylamine hydrochloride (0.3 M) and imidazole (0.225 M) in a NMP / CH₂Cl₂ mixture¹ (5:1, 20 min, 3 mL, ×3) is performed before the capping step.

Syntheses starting from Fmoc-Gly-Wang or Rink polystyrene resins were performed on the 433A synthesizer, using the 0.1 mmol scale Fastmoc program purchased from the manufacturer, with a single coupling with HCTU followed by capping.

The crude peptide was deprotected and cleaved from the resin through a treatment with TFA/H₂O/iPr₂SiH/phenol, 88/5/2/5 for 2 h, and the peptide was precipitated by dilution into an ice-cold 1:1 diethyl ether/petroleum ether mixture, recovered by centrifugation and washed twice with diethyl ether.

¹ Diaz-Mochon J. J., Bialy L., Bradley M., Org. Lett., 2004, 6, 7, 1127-1129
3- Optimization of the benzyl group to maximize the N-acylation yield

3a- Synthesis of cysteinyl peptide resin 1

Supplementary scheme S1: Synthesis of peptide resin 1.

Peptide resin 1 was obtained through automated SPPS (protocol p S3) starting from Fmoc-Gly-Wang resin (130 mg, 0.79 mmol/g, 0.1 mmol). An aliquot of the resin was cleaved (protocol p S3) in order to characterize the corresponding peptide S1.

S1:

MALDI-TOF MS (m/z): [M+H]^+ calcd for C_{29}H_{47}N_{9}O_{8}S: 682.3, found: 682.3.

HPLC: retention time: 14.41 min (Nucleosil, gradient: 5-50 % B/A over 30 min).

Supplementary figure S1: HPLC trace of crude S1.
3b- Synthesis of peptide resins 2a-d by solid-supported N-alkylation

Peptide resin 1 (10 µmol) was introduced into a polypropylene syringe fitted with a polypropylene frit and a PTFE stopcock, then swollen in a DMF/MeOH/AcOH (9:9:2) mixture for 5 min. The syringe was drained and the resin was washed with a DMF/MeOH (1:1) mixture. The appropriate aldehyde (100 µmol, 10 equiv.) dissolved in DMF/MeOH (0.4 mL) was added then the syringe was left for 45 min under gentle stirring. The syringe was drained off and the resin was washed with DMF/MeOH. Without delay, sodium cyanoborohydride (13 mg, 200 µmol, 20 equiv.) dissolved in DMF/MeOH/AcOH (0.4 mL) was added and the syringe was left for 30 min under gentle stirring. The syringe was drained and the resin was thoroughly washed with DMF/MeOH/AcOH, NMP, 5% tPr₂NEt in NMP then NMP. An aliquot of each peptide resin 2a-c was cleaved (protocol p S3) in order to characterize the corresponding peptides S2a-c.

S2a:

MALDI-TOF (m/z): [MH]+ calcd for C₃₇H₅₅N₉O₁₀S: 818.4, found: 818.4.

HPLC: retention time: 19.59 min (Nucleosil, gradient: 5-50 % B/A over 30 min).
Supplementary figure S2: HPLC trace of crude S2a.

S2b:

MALDI-TOF (m/z): [MH]+ calcd for C_{36}H_{53}N_{9}O_{9}S: 788.4, found: 788.3.

HPLC: retention time: 19.17 min (Nucleosil, gradient: 5-50 % B/A over 30 min).

Supplementary figure S3: HPLC trace of crude S2b.
**S2c:**

**MALDI-TOF** \( (m/z): [MH]^+ \) calcd for \( C_{36}H_{52}N_{10}O_{11}S: 833.4 \), found: 833.3.  

**HPLC:** retention time: 20.44 min (Nucleosil, gradient: 5-50 % B/A over 30 min).  

**Supplementary figure S4:** HPLC trace of crude S2c.  

**Supplementary scheme S3:** Synthesis of \( N \)-ethyl peptide resin 2d through solid-supported reductive amination.
Peptide resin 1 (10 µmol, 1 equiv.) was introduced into a polypropylene syringe fitted with a polypropylene frit and a PTFE stopcock and swollen in a mixture of DMF/MeOH/AcOH (9:9:2) for 5 min. The syringe was drained and the resin was washed with a mixture of DMF/MeOH (1:1). Acetaldehyde (100 µmol, 10 equiv.) dissolved in DMF/MeOH (0.4 mL) was added then the syringe was left for 5 min under gentle stirring. The syringe was drained and the resin was washed with DMF/MeOH. Without delay, sodium cyanoborohydride (13 mg, 200 µmol, 20 equiv.) dissolved in DMF/MeOH/AcOH (0.4 mL) was added and the syringe was left for 30 min under gentle stirring. The syringe was drained and the resin was thoroughly washed with DMF/MeOH/AcOH, NMP, 5% iPr₂NEt in NMP then NMP. An aliquot of the resulting peptide resin was cleaved (protocol p S3) in order to characterize N-ethyl peptide S2d.

**S2d:**

**MALDI-TOF (m/z):** [MH]+ calcd for C₃₁H₅₁N₉O₈S: 710.4, found 710.4.

**HPLC:** retention time: 15.36 min (Nucleosil, gradient: 5-50 % B/A over 30 min).

Supplementary figure S5: HPLC trace of crude S2d.
3c- Study on the solid-supported N-acylation of secondary amines 2a-d

General procedure for solid-supported N-acylation

Peptide resin (2a-d) was introduced into a polypropylene syringe fitted with a polypropylene frit and a PTFE stopcock and swollen with NMP. Then, Fmoc-Gly-OH (10 equiv.), HBTU (9.5 equiv.) and HOBT (9.5 equiv.) were dissolved in NMP (0.1 M final concentration in amino acid) prior to addition of iPr₂NEt (20 equiv.). The resulting solution was immediately added to the resin. The syringe was left for 2 h under gentle stirring, then the syringe was drained and the resin was washed with NMP. An aliquot resin was cleaved using the standard procedure (p S3) to determine the coupling yields and characterize the corresponding N-acylated peptides.

Supplementary scheme S4: Study on the influence of the N-alkyl group (R) of peptide resins 2a-d on the N-acylation yield.

3a:

MALDI-TOF (m/z): [MH]⁺ calcd for C₃₉H₅₈N₁₀O₁₁S: 875.4, found: 875.4.

HPLC: retention time: 17.24 min (Nucleosil, isocratic 17% B/A over 30 min at 50°C).
**Supplementary figure S6: HPLC trace of crude 3a.**

**3b:**

**MALDI-TOF** \((m/z): [MH]^+ \text{ calc'd for C}_{38}\text{H}_{56}\text{N}_{10}\text{O}_{10}\text{S}: 845.4, \text{ found 845.4.}\
**HPLC:** retention time: 19 min (Nucleosil, gradient: 5-50 % B/A over 30 min).

**Supplementary figure S7: HPLC trace of crude 3b.**
**3c:**

MALDI-TOF (m/z): [MH]+ calcd for C_{38}H_{55}N_{11}O_{12}S: 890.4, found: 890.4.

HPLC: retention time: 20.53 min (Nucleosil, gradient: 5-50 % B/A over 30 min).

![Supplementary figure S8: HPLC trace of crude 3c.](image)

**N-acylation of peptide resin 2d using HBTU/HOBt followed by deprotection of the Fmoc group then cleavage of the resin did not show any detectable amount of the expected N-acylated peptide 3d. For characterization purpose, the N-acylation was performed using HATU as the coupling reagent, yielding to a small amount of 3d together with the non-acylated peptide S2d.**

![Supplementary scheme S5: N-acylation of peptide resin 2d using HATU as the coupling reagent.](image)
3d:
MALDI-TOF (m/z): [MH]+ calcd for C_{33}H_{54}N_{10}O_{9}S: 767.4, found 767.4.
HPLC: retention time: 17.02 min (Nucleosil, gradient: 5-50% B/A over 30 min).

4- Evaluation of the phenol pKa of an N-acyl-N-(Hnb)Cys compound

Synthesis of S-alkylated compound S3

In order to prevent any N-S shift that would make uncertain the determination of the pKa of the phenol group, we synthesized a model dipeptide S3 S-alkylated with an acetamidomethyl (Acm) protective group.

Fmoc-Rink polystyrene resin (182 mg, 0.55 mmol/g, 0.1 mmol) was introduced into a polypropylene syringe fitted with a polypropylene frit and a PTFE stopcock and swollen with NMP. The resin was treated with 20% piperidine in NMP (10 mL, 3 × 3 min) and washed with NMP. Then, Fmoc-Cys(Acm)-OH (414 mg, 1 mmol, 10 equiv.), HBTU (360 mg, 0.95 mmol, 9.5 equiv.) and HOBT (128 mg, 0.95 mmol, 9.5 equiv.)

Supplementary scheme S6: Synthesis of the model dipeptide S3.
were dissolved in NMP (10 mL) prior to addition of $iPr_2$NEt (348 µL, 2 mmol, 20 equiv.). The resulting solution was immediately added to the resin. The syringe was left for 1 h under gentle stirring, then the syringe was drained and the resin was washed with NMP. Fmoc group was deprotected by three successive treatments with 20% piperidine in NMP (10 mL) for 3 min. The 2-hydroxy-5-nitrobenzyl linker was then introduced following the general procedure (p S5). Fmoc-Ala-OH (311 mg, 1 mmol, 10 equiv.) was coupled following the same coupling procedure as described above. Coupling was repeated once. The resin was then treated with 20% piperidine in NMP (10 mL, 3 × 3 min), washed with NMP, then treated with acetic anhydride (571 µL, 6 mmol, 60 equiv.), $iPr_2$NEt (272 µL, 0.39 mmol, 15.5 equiv.) and HOBt (24 mg, 0.044 mmol, 1.8 equiv.) in NMP (12 mL) for 2 × 1 h. Peptide-resin was then treated with 20% piperidine in NMP (10 mL, 3 × 3 min), washed with NMP then CH₂Cl₂, and finally cleaved using a solution of TFA / H₂O (9:1) for 2 h. The solvents were removed under reduced pressure to give compound S₃.

S₃:
ESI-HRMS ($m/z$): [MH]$^+$ calcd for $C_{18}H_{26}N_5O_7$: 456.1547, found: 456.1544.
HPLC: retention time: 12.35 min (Nucleosil, gradient: 5-95 % B/A over 30 min).

![Graph](image)

Supplementary figure S10: HPLC trace of crude S₃.

_Determination of the phenol pKa of S-alkylated compound S₃_

The pKa of the phenol group was determined by acid-base titration of a dilute (10 µM) solution of compound S₃ in Milli-Q water. The pH was adjusted with 1M HCl and NaOH solutions. The titration was followed by UV spectrophotometry on an Uvikon 923 double beam UV/VIS spectrophotometer. The results were plotted at the $\lambda_{max}$ of
the phenol and phenolate species (determined to be 320 and 407 nm, respectively) against pH.

**Supplementary figure S11:** UV absorbance of compound S3 as a function of pH.

The pKa value (6.5 ± 0.1) was determined fitting with a sigmoidal function and measuring the inflection point of the curve.

407 nm: \( y = 0.36324 + (0.00891 \cdot 0.36324)/(1 + \exp((x-6.51822)/0.59329)) \) \( R^2 = 0.996 \)

320 nm: \( y = 0.0378 + (0.19762 \cdot 0.0378)/(1 + \exp((x-6.53917)/0.60827)) \) \( R^2 = 0.993 \)

**5- Characterization of a model N-acyl-N-(Hnb)Cys compound**

5a- \(^1\)H and \(^{13}\)C NMR of S5 and comparison with \(^1\)H NMR of O-methylated S6

**Supplementary scheme S7:** Synthesis of the compound S5.

Fmoc-protected Rink polystyrene resin (63.3 mg, 0.79 mmol/g, 0.05 mmol) was introduced into a polypropylene syringe fitted with a polypropylene frit and a PTFE stopcock and swollen with NMP. Fmoc group was deprotected by three successive treatments with 20% piperidine in NMP (5 mL) for 3 min. Then, Fmoc-Cys(SfBu)-OH (216 mg, 0.5 mmol, 10 equiv.) and HCTU (186 mg, 0.45 mmol, 9 equiv.) were
dissolved in NMP (1 mL) prior to addition of iPr$_2$NEt (175 µL, 1 mmol, 20 equiv.). The resulting solution was immediately added to the resin. The syringe was left for 1 h under gentle stirring, then the syringe was drained and the resin was washed with NMP. Fmoc group was removed by three successive treatments with 20% piperidine in NMP (5 mL) for 3 min to give resin S4. The 2-hydroxy-5-nitrobenzyl linker was then introduced following the general procedure (p S5 for 0.05 mmol). Then, acetic acid (30 µL, 0.5 mmol, 10 equiv.) and HCTU (186 mg, 0.45 mmol, 9 equiv.) were dissolved in NMP (1 mL) prior to addition of iPr$_2$NEt (175 µL, 1 mmol, 20 equiv.). The resulting solution was immediately added to the resin. The syringe was left for 1 h under gentle stirring, then the syringe was drained and the resin was washed with NMP. Amino acid-resin was then treated with 20% piperidine in NMP (5 mL, 3 × 3 min), washed with NMP then CH$_2$Cl$_2$. Finally, the compound S5 was obtained after treatment with TFA/H$_2$O/iPr$_3$SiH, 93/5/2 (4 mL) for 2 h and characterized. Note that the hydrolysis of the C-terminal amide into the acid carboxylic was quantitative under the cleavage conditions. The product was purified by flash reverse phase chromatography (45 mg crude in 1 mL MeCN, column: RP18 25-40 µm - 20 g, Götec-Labortechnik GmbH, gradient: 30-50% B/A over 20 min, 35 mL/min).

\[ \text{S5:} \]

ESI-HRMS ($m/z$): [MH]$^+$ calcd for C$_{16}$H$_{23}$N$_2$O$_6$S$_2$: 403.0998, found: 403.0993.

HPLC: retention time: 28.36 min (Nucleosil, gradient: 20-50% B/A over 30 min).

Supplementary figure S12: HPLC trace of crude S5.
Pure S5 has been analyzed by 1H NMR in DMSO-$d_6$. Two conformers are observed: trans and cis isomers (evaluated ratio 73:27). On the supplementary figure S14, arrows indicate observed NOE on a ROESY 2D spectrum (mixing time = 200 ms) used to characterize the two conformers.

**Supplementary figure S14: Cis and trans isomers of S5 and observed ROESY connectivities.**

**Trans isomer of S5**

1H NMR (600 MHz, DMSO-$d_6$): 13.40 (1H, H$_3$, s), 11.48 (1H, H$_{10}$, s), 8.42 (1H, H$_7$, d, $J = 2.8$ Hz), 8.10 (1H, H$_8$, dd, $J = 8.9,2.9$ Hz), 7.01 (1H, H$_9$, d, $J = 8.9$ Hz), 4.64-5.52 (2H, H$_{6a}$ and H$_{6b}$, m), 4.26 (1H, H$_2$, t, $J = 6.7$ Hz), 3.40-3.36 (1H, H$_{4b}$, m), 2.87 (1H, H$_{4a}$, dd, $J = 13.5,7.5$ Hz), 2.06 (3H, H$_1$, s), 1.21 (9H, H$_5$, s).
**Cis isomer of S5**

1H NMR (600 MHz, DMSO-d$_6$): 12.74 (1H, H$_3$, br), 11.32 (1H, H$_{10}$, s), 8.04-8.00 (1H, H$_8$, m), 8.02 (1H, H$_7$, br), 6.95 (1H, H$_9$, d, J = 8.7 Hz), 4.85 (1H, H$_2$, dd, J = 9.6,4.8 Hz), 4.55-4.52 (1H, H$_{6b}$, m), 4.18 (1H, H$_{6a}$, d, J = 16.6 Hz), 3.26 (1H, H$_{4b}$, dd, J = 14,4.8 Hz), 3.01 (1H, H$_{4a}$, dd, J = 13.9,9.6 Hz), 2.22 (3H, H$_1$, s), 1.24 (9H, H$_5$, s).

**Trans and cis isomers of S5 - 27 13C peaks observed** (over 28, the remaining signal probably being hidden by another peak at the same chemical shift and not discernable at the 600 MHz resolution)

13C NMR (100 MHz, DMSO-d$_6$): 171.72, 170.85, 170.80, 161.75, 160.80, 139.63, 139.39, 125.27, 125.16, 125.12, 124.65, 124.54, 124.21, 115.15, 114.98, 99.51, 60.38, 59.42, 48.37, 48.18, 47.60, 45.76, 40.16, 40.06, 29.47, 29.42, 22.04, 21.59.
Supplementary figure S15: Copy of the $^1$H NMR spectrum of S5.
Supplementary figure S16: Copy of the $^1$H NMR spectrum (zoom) of S5.
Supplementary figure S17: Copy of the $^{13}$C NMR spectrum of **S5**.
Supplementary scheme S8: Synthesis of the compound S6.

Fmoc-protected Rink polystyrene resin (63.3 mg, 0.79 mmol/g, 0.05 mmol) was introduced into a polypropylene syringe fitted with a polypropylene frit and a PTFE stopcock and swollen with NMP. Fmoc group was deprotected by three successive treatments with 20% piperidine in NMP (5 mL) for 3 min. Then, Fmoc-Cys(SiBu)-OH (216 mg, 0.5 mmol, 10 equiv.) and HCTU (186 mg, 0.45 mmol, 9 equiv.) were dissolved in NMP (1 mL) prior to addition of iPr2NEt (175 µL, 1 mmol, 20 equiv.). The resulting solution was immediately added to the resin. The syringe was left for 1 h under gentle stirring, then the syringe was drained and the resin was washed with NMP. Fmoc group was removed by three successive treatments with 20% piperidine in NMP (5 mL) for 3 min to give resin S4. The 2-hydroxy-5-nitrobenzyl linker was then introduced following the general procedure (p S5 for 0.05 mmol). Then, acetic acid (30 µL, 0.5 mmol, 10 equiv.) and HCTU (186 mg, 0.45 mmol, 9 equiv.) were dissolved in NMP (1 mL) prior to addition of iPr2NEt (175 µL, 1 mmol, 20 equiv.). The resulting solution was immediately added to the resin. The syringe was left for 1 h under gentle stirring, then the syringe was drained and the resin was washed with NMP. Amino acid-resin was then treated with 20% piperidine in NMP (5 mL, 3 × 3 min), washed with NMP then CH2Cl2. Methylation of the phenol group was performed using a solution of Mel (50 equiv.), iPr2NEt (50 equiv.) in DMF for 4 h and the resin was washed with NMP and CH2Cl2. Finally, the compound S6 was obtained after treatment with TFA/H2O/iPr3SiH, 93/5/2 (4 mL) for 2 h and characterized. Note that the hydrolysis of the C-terminal amide into the acid carboxylic was quantitative under the cleavage conditions. The product was purified by semi-preparative HPLC (Nucleosil, 40-55 % B/A over 15 min).

S6:
ESI (m/z): [MH]+ calcd for C17H25N2O6S2: 417.1, found: 417.1.
HPLC: retention time: 31.28 min (Nucleosil, gradient: 20-50% B/A over 30 min).
Supplementary figure S18: HPLC trace of pure S6.

Pure S6 has been analyzed by $^1$H NMR in DMSO-$d_6$ for comparison of the cis-trans ratio with S5. Two conformers are observed: trans and cis isomers (evaluated ratio 72:28, nearly identical with S5 ratio). On the supplementary figure S19, arrows indicate observed NOE on a ROESY 2D spectrum (mixing time = 200 ms) used to characterize the two conformers.

**Supplementary figure S19: Cis and trans isomers of S6 and observed ROESY connectivities.**

**Trans isomer (major) of S6**

$^1$H NMR (600 MHz, DMSO-$d_6$): 12.77 (1H, H$_3$, bs), 8.46 (1H, H$_7$, d, $J = 2.7$ Hz), 8.26 (1H, H$_8$, dd, $J = 9.1,2.8$ Hz), 7.26 (1H, H$_9$, d, $J = 9.1$ Hz), 4.6 (2H, H$_{6a}$ and H$_{6b}$, dd, $J = 45.3,17.3$ Hz), 4.25 (1H, H$_2$, t, $J = 6.5$ Hz), 3.96 (3H, H$_{10}$, s), 3.36 (1H, H$_{4b}$, dd, $J = 13.5,5.8$ Hz), 2.91 (1H, H$_{4a}$, dd, $J = 13.7,7.8$ Hz), 2.04 (3H, H$_1$, s), 1.22 (9H, H$_5$, s).

**Cis isomer (minor) of S6**

$^1$H NMR (600 MHz, DMSO-$d_6$): 13.43 (1H, H$_3$, bs), 8.16 (1H, H$_8$, dd, $J = 9.0,2.9$ Hz), 8.03 (1H, H$_7$, d, $J = 2.7$ Hz), 7.19 (1H, H$_9$, d, $J = 9.1$ Hz), 4.86-4.79 (1H, H$_2$, m), 4.52 (1H, H$_{6b}$, t, $J = 16.9$ Hz), 4.19 (1H, H$_{6a}$, d, $J = 17.4$ Hz), 3.94 (3H, H$_{10}$, s), 3.29-3.25 (1H, H$_{4b}$, m), 3.02-2.99 (1H, H$_{4a}$, m), 2.21 (3H, H$_1$, s), 1.25 (9H, H$_5$, s).
Supplementary figure S20: 1H NMR of S6.
5b- Molar extinction coefficient of the Hnb group of S5

The molar extinction coefficient ($\varepsilon$) of the Hnb group of S5 was determined by UV spectroscopy at 275, 280, 320 nm in a H$_2$O/MeCN/TFA (8:2:0.001) solution.

| Wavelength (nm) | 275 | 280 | 320 |
|-----------------|-----|-----|-----|
| $\varepsilon$ (L.mol$^{-1}$.cm$^{-1}$) | 1846 | 2359 | 6325 |

Supplementary table S1: Molar extinction coefficient of S5.

Supplementary figure S21: UV spectrum of S5.

6- Optimization of the cysteine thiol protecting group

6a- Synthesis and stability of (S-Trt) model peptide S7

Supplementary scheme S9: Synthesis of the model peptide S7 showing hydrolysis co-product S8.

Fmoc-protected Rink polystyrene resin (126.6 mg, 0.79 mmol/g, 0.1 mmol) was introduced into a polypropylene syringe fitted with a polypropylene frit and a PTFE stopcock and swollen with NMP. Fmoc group was deprotected by three successive treatments with 20% piperidine in NMP (10 mL) for 3 min. Then, Fmoc-Cys(Trt)-OH
(586 mg, 1 mmol, 10 equiv.) and HCTU (372 mg, 0.90 mmol, 9 equiv.) were dissolved in NMP (2 mL) prior to addition of iPr₂NEt (348 µL, 2 mmol, 20 equiv.). The resulting solution was immediately added to the resin. The syringe was left for 1 h under gentle stirring, then the syringe was drained and the resin was washed with NMP. Fmoc group was removed by three successive treatments with 20% piperidine in NMP (10 mL) for 3 min. The 2-hydroxy-5-nitrobenzyl linker was then introduced following the general procedure (p S5). The Ac-LYRAA sequence was installed by standard SPPS procedure (protocol p S3), an extended double coupling (2 × 2 h) was performed for the first alanine and a normal double coupling (2 × 30 min) was performed for the arginine. Peptide resin was then treated with 20% piperidine in NMP (10 mL, 3 × 3 min), washed with NMP then CH₂Cl₂, and finally cleaved following the general procedure p S3 to give compound S7 and side product S8 (89:11) (Measured by HPLC at 275 nm). Peptides were obtained as a mixture of C-terminal amide and acid arising from hydrolysis of the amide under the TFA cleavage conditions.² Their corresponding thioester forms arising from a premature N-S shift was also observed (compounds S7’ and S8’).

**S7:**
**ESI-HRMS (m/z):** [MH]+ calcd for C₃₉H₅₈N₁₁O₁₁S: 888.4038, found: 888.4028.
**HPLC:** retention time: 4.36 min (Chromolith, gradient: 5-50 % B/A over 5 min).

**S8:**
**ESI-HRMS (m/z):** [MH]+ calcd for C₃₉H₅₇N₁₀O₁₂S: 889.3878, found: 889.3881.
**HPLC:** retention time: 4.44 min (Chromolith, gradient: 5-50 % B/A over 5 min).

**S7’:**
**ESI-HRMS (m/z):** [MH]+ calcd for C₃₉H₅₈N₁₁O₁₁S: 888.4035, found: 888.4028.
**HPLC:** retention time: 3.82 min (Chromolith, gradient: 5-50 % B/A over 5 min).

**S8’:**
**ESI-HRMS (m/z):** [MH]+ calcd for C₃₉H₅₇N₁₀O₁₂S: 889.3884, found: 889.3881.
**HPLC:** retention time: 3.92 min (Chromolith, gradient: 5-50 % B/A over 5 min).

---
² a) C. J. Creighton, T. T. Romoff, J. H. Bu, M. Goodman, *J. Am. Chem. Soc.* 1999, 121, 6786–6791; b) M. Teixidó, F. Albericio, E. Giralt, *J. Peptide Res.* 2005, 65, 153–166.
Supplementary figure S22: HPLC trace of crude S7.

Supplementary figure S23: LC/MS analysis of crude S7. Blue trace UV 214 nm, red trace TIC.

| Peak (tr (min)) | [MH]$^+$ (m/z) Calc. | [MH]$^+$ (m/z) found | Attributed to                                      |
|-----------------|----------------------|----------------------|---------------------------------------------------|
| 15 (11.24)      | 888.4038             | 888.4035             | Premature N→S shift of S7 (thioester form S7')   |
| 17 (11.77)      | 889.3878             | 889.3884             | Premature N→S shift of S8 (thioester form S8')   |
| 25 (13.40)      | 888.4038             | 888.4028             | S7                                                |
| 26 (13.55)      | 889.3878             | 889.3881             | S8                                                |

Supplementary table S2: Attribution of selected peaks observed in LC/MS analysis of crude S7.
To test the stability of this mixture of peptides, 1 mg of crude product was solubilized in 1 mL of a 8:2:0.01 H₂O/MeCN/TFA mixture. The stability of S7 was studied by analyzing this solution by LC-MS after overnight incubation. Formation of Ac-LYRAA-OH was observed, probably arising from hydrolysis of S7' and S8'.

Supplementary figure S24:
LC/MS trace of crude S7 dissolved in 8:2:0.01 H₂O/MeCN/TFA (1mg/mL), after t = 17.5 h incubation at room temperature. Blue trace UV 214 nm, red trace TIC.

Supplementary table S3: Attribution of selected peaks observed in LC/MS analysis of crude S7 after 17.5 h in solvents A/B.

| Peak (tr (min)) | [MH]+ (m/z) Calc. | [MH]+ (m/z) found | Attributed to |
|-----------------|-------------------|-------------------|--------------|
| 5 (9.76)        | 635.3517          | 635.3516          | Ac-LYRAA-OH  |
| 10 (11.72)      | 888.4038          | 888.4040          | Premature N→S shift of S7 (thioester form S7') |
| 12 (12.21)      | 889.3878          | 889.3888          | Premature N→S shift of S8 (thioester form S8') |
| 20 (13.75)      | 888.4038          | 888.4028          | S7            |
| 22 (13.90)      | 889.3878          | 889.3881          | S8            |
6b- Synthesis and stability of (S-SfBu) model peptide 7

Supplementary scheme S10: Synthesis of model peptide 7.

Fmoc-protected Rink polystyrene resin (126.6 mg, 0.79 mmol/g, 0.1 mmol) was introduced into a polypropylene syringe fitted with a polypropylene frit and a PTFE stopcock and swollen with NMP. Fmoc group was removed by three successive treatments with 20% piperidine in NMP (10 mL) for 3 min. Then, Fmoc-Cys(SfBu)-OH (432 mg, 1 mmol, 10 equiv.) and HCTU (372 mg, 0.90 mmol, 9 equiv.) were dissolved in NMP (2 mL) prior to addition of iPr2NEt (348 µL, 2 mmol, 20 equiv.). The resulting solution was immediately added to the resin. The syringe was left for 1 h under gentle stirring, then the syringe was drained and the resin was washed with NMP. Fmoc group was removed by three successive treatments with 20% piperidine in NMP (10 mL) for 3 min. The 2-hydroxy-5-nitrobenzyl linker was then introduced following the general procedure (p S5). The Ac-LYRAA sequence was installed by standard SPPS procedure (protocol p S3), an extended double coupling (2 × 2h) was performed for the first alanine and a normal double coupling (2 × 30 min) was performed for the arginine. Peptide resin was then treated with 20% piperidine in NMP (10 mL, 3 × 3 min), washed with NMP then CH2Cl2, and finally cleaved following the general procedure p S3 to give compound 7. Peptide was obtained as a mixture of C-terminal amide and acid arising from hydrolysis of the amide under the TFA cleavage conditions.2

MALDI-TOF (m/z): [MH]+ calcd for C43H66N11O11S2: 976.44, found: 976.45.
HPLC: retention time: 30.89 min (Nucleosil, gradient: 5-50% B/A over 30 min).

7:
To test the stability of this mixture of peptides, 1 mg of crude product was solubilized in 1 mL of 8:2:0.01 H₂O/MeCN/TFA. The stability of 7 was studied by analyzing this solution by HPLC at different times. Those products are perfectly stable in acidic aqueous conditions.

Supplementary figure S26: HPLC trace of crude 7 dissolved in 8:2:0.01 H₂O/MeCN/TFA (1mg/mL), after t = 17.5 h incubation at room temperature.
7- Optimization of the spacer between the cysteine and the resin

7a- Direct attachment to Rink linker – amide hydrolysis co-product

Supplementary scheme S11: Synthesis of the model peptide 7.

During the cleavage step (using procedure p S3) in order to obtain peptide 7, the formation of peptide 8 was also observed (12 % determined by HPLC integration at 275 nm) from the hydrolysis of the C-terminal amide function into a C-terminal carboxylic acid.

**7:**
MALDI-TOF (m/z): [MH]+ calcd for C_{43}H_{66}N_{11}O_{11}S_{2}: 976.44, found: 976.45.
HPLC: retention time: 30.89 min (Nucleosil, gradient: 5-50% B/A over 30 min).

**8 – C-terminal hydrolysis co-product:**
MALDI-TOF (m/z): [MH]+ calcd for C_{43}H_{65}N_{10}O_{12}S_{2}: 977.42, found: 977.43.
HPLC: retention time: 31.27 min (Nucleosil, gradient: 5-50% B/A over 30 min).

**Supplementary figure S27:** HPLC trace of crude 7 showing hydrolysis side product 8.
S9: Direct attachment to PHB Tentagel resin (Wang type linker)

Supplementary scheme S12: Formation of the C-terminal piperidine adduct S9.³

Pre-loaded Fmoc-Cys(SfBu)-PHB Tentagel resin (527 mg, 0.19 mmol/g, 0.1 mmol) was introduced into a polypropylene syringe fitted with a polypropylene frit and a PTFE stopcock and swollen with NMP. Fmoc group was removed by three successive treatments with 20% piperidine in NMP (10 mL) for 3 min. The 2-hydroxy-5-nitrobenzyl linker was then introduced following the general procedure (p S5). For a 10 µmol aliquot, acetic acid (6 µL, 0.1 mmol, 10 equiv.) and HCTU (37.2 mg, 0.09 mmol, 9 equiv.) were dissolved in NMP (0.2 mL) prior to addition of iPr₂NEt (35 µL, 0.2 mmol, 20 equiv.). The resulting solution was immediately added to the resin. The syringe was left for 1 h under gentle stirring, then the syringe was drained and the resin was washed with NMP. Amino acid-resin was then treated with 20% piperidine in NMP (1 mL, 3 × 3 min), washed with NMP then CH₂Cl₂. Finally, the product was cleaved using TFA / H₂O (95:5) for 2h. The solvents were removed by evaporation and analysis showed the complete formation of the C-terminal piperidine adduct S9 and no trace of the expected Cys(SfBu) compound.

S9: 
ESI-HRMS (m/z): [MH]+ calcd for C₁₇H₂₄N₃O₆: 366.1665, found: 366.1662. 
HPLC: retention time: 6.50 min (Nucleosil, gradient: 20-50% B/A over 30 min).

³ Lukszo J., Patterson D., Albericio F., Kates S. A., Letters in Peptide Science, 1996, 3, 157-166
7c- Introduction of a Gly residue as a spacer

Tentagel R resin (476 mg, 0.21 mmol/g, 0.1 mmol) was introduced into a polypropylene syringe fitted with a polypropylene frit and a PTFE stopcock and swollen with NMP. Fmoc group was removed by three successive treatments with 20% piperidine in NMP (10 mL) for 3 min. Then, Fmoc-Rink-OH (270 mg, 0.5 mmol, 5 equiv.) and HATU (190 mg, 0.5 mmol, 5 equiv.) were dissolved in NMP (2 mL) prior to addition of iPr2NEt (175 µL, 1 mmol, 10 equiv.). The resulting solution was immediately added to the resin. The syringe was left for 1.5 h under gentle stirring, then the syringe was drained and the resin was washed with NMP. Fmoc group was removed by three successive treatments with 20% piperidine in NMP (10 mL) for 3 min. Fmoc-Gly-OH (297 mg, 1 mmol, 10 equiv.) and HCTU (372 mg, 0.90 mmol, 9 equiv.) were dissolved in NMP (2 mL) prior to addition of iPr2NEt (348 µL, 2 mmol, 20 equiv.). The resulting solution was immediately added to the resin. The syringe was left for 1 h under gentle stirring, then the syringe was drained and the resin was washed.
washed with NMP. Then, Fmoc group was removed by three successive treatments with 20% piperidine in NMP (10 mL) for 3 min. Fmoc-Cys(StBu)-OH (432 mg, 1 mmol, 10 equiv.) and HCTU (372 mg, 0.90 mmol, 9 equiv.) were dissolved in NMP (10 mL) prior to addition of iPr₂NEt (348 µL, 2 mmol, 20 equiv.). The resulting solution was immediately added to the resin. The syringe was left for 1.5 h under gentle stirring, then the syringe was drained and the resin was washed with NMP. Fmoc group was removed by three successive treatments with 20% piperidine in NMP (10 mL) for 3 min. The 2-hydroxy-5-nitrobenzyl linker was then introduced following the general procedure (p S5). The Ac-LYRAA sequence was installed by standard SPPS procedure (protocol p S3), an extended double coupling (2 × 2h) was performed for the first alanine and a normal double coupling (2 × 30 min) was performed for the arginine. Peptide resin was then treated with 20% piperidine in NMP (10 mL, 3 × 3 min), washed with NMP then CH₂Cl₂, and finally cleaved following the general procedure p S3 to give compound 9. The product was purified by HPLC (Nucleosil, 40-45 % B/A over 10 min).

9: MALDI-TOF (m/z): [MH]+ calcd for C₄₅H₆₉N₁₂O₁₂S₂: 1033.45, found: 1033.49. 
HPLC: retention time: 8.70 min (Chromolith, gradient: 5-50% B/A over 10 min).

Supplementary figure S29: HPLC trace of crude 9.
**Supplementary figure S30: HPLC trace of pure 9.**

8- **N-Acylation with the 20 different proteogenic amino acids**

**Supplementary scheme S14: Synthesis of (Hnb)-dipeptide 4.**

The Rink linker, Gly and Cys(S/Bu) were installed through automated SPPS (protocol p S3) starting from Tentagel R resin (120 mg, 0.21 mmol/g, 25 µmol) in order to obtain peptide resin S10.

**Automated reductive amination procedure for a peptide synthesizer**

Peptide resin S10 (25 µmol) was introduced into a Prelude synthesizer reactor, washed two times with 3 mL of a DMF/MeOH (1:1) mixture for 30 sec, then swollen in 3 mL of a DMF/MeOH/AcOH (9:9:2) mixture for 5 min. The reactor was drained off and the resin was washed four times with 3 mL of a DMF/MeOH (1:1) mixture for 30 s. 2 mL of a solution of 2-hydroxy-5-nitrobenzaldehyde in DMF/MeOH (1:1) (125 mM, 10 equiv.) were added then the reactor was left for 1h under stirring by nitrogen bubbling. The reactor was drained off and the resin was washed four times with 3 mL of DMF/MeOH (1:1) for 15 sec. Without delay, 2 mL of a fresh solution of sodium
cyanoborohydride in a DMF/MeOH/AcOH (9:9:2) mixture (250 mM, 20 equiv.) were added and the reactor was left for 1 h under stirring by nitrogen bubbling. The reactor was drained and the resin was washed with DMF/MeOH (1:1) (3 mL, 30 sec, × 4), NMP (3 mL, 30 s, × 3), 20% piperidine in NMP (3 mL, 30 s, × 3), NMP (3 mL, 30 sec, × 3), dichloromethane (5 mL, 30 s, × 3) and NMP (3 mL, 30 s, × 2).

### Resin conditioning

| Operation       | Reagent/Solvent          | Volume       | Time         |
|-----------------|--------------------------|--------------|--------------|
| 1) DMF/MeOH wash| DMF/MeOH (1:1)           | 3 mL × 2     | 0.5 min × 2  |
| 2) DMF/MeOH/AcOH wash | DMF/MeOH/AcOH (9:9:2) | 3 mL        | 5 min        |
| 3) DMF/MeOH wash | DMF/MeOH (1:1)           | 3 mL × 4     | 0.5 min × 4  |

### Imine formation

| Operation                                      | Reagent/Solvent          | Volume   | Time         |
|-----------------------------------------------|--------------------------|----------|--------------|
| 1) Add 2-hydroxy-5-nitrobenzaldehyde (Hnba)  | 125 mM Hnba in DMF/MeOH (1:1) | 2 mL     | 60 min       |
| 2) DMF/MeOH wash                              | DMF/MeOH (1:1)           | 3 mL × 4 | 0.25 min × 4 |

### Reduction

| Operation          | Reagent/Solvent          | Volume       | Time         |
|--------------------|--------------------------|--------------|--------------|
| 1) Add NaBH$_3$CN  | 250 mM NaBH$_3$CN in DMF/MeOH/AcOH (9:9:2) | 2 mL       | 60 min       |
| 2) DMF/MeOH wash   | DMF/MeOH (1:1)           | 3 mL × 4     | 0.5 min × 4  |
| 3) NMP wash        | NMP                      | 3 mL × 3     | 0.5 min × 3  |
| 4) Base wash       | 20% piperidine in NMP    | 3 mL × 3     | 3 min × 3    |
| 5) NMP wash        | NMP                      | 3 mL × 3     | 0.5 min × 3  |
| 6) Dichloromethane wash | CH$_2$Cl$_2$       | 5 mL × 3     | 0.5 min × 3  |
| 7) NMP Wash        | NMP                      | 3 mL × 2     | 0.5 min × 2  |

### N-acylation yield determination

The 20 different proteogenic amino acids were then coupled on 4 using a single standard automated coupling on the prelude (protocol p S3), after that the peptide resins were treated with 20% piperidine in NMP (3 × 3 min); finally, an aliquot (5 µmol) of each resins was cleaved using 1 mL of a TFA/H$_2$O/iPr$_3$SiH (93:5:2) solution for 2 h. After evaporation of TFA, the samples were analyzed by HPLC; the N-acylation yields were quantified by integration at 315 nm, not taking into account eventual differences in molar extinction coefficient of the products.
Non acylated product - S11:

**ESI-HRMS** \(m/z\): [MH]\(^+\) calcd for C\(_{16}\)H\(_{25}\)N\(_4\)O\(_5\)S\(_2\): 417.1266, found: 417.1261.

**HPLC**: retention time: 2.12 min (Chromolith, gradient: 20-50 % B/A over 5 min).

Ala - 5’c:

**ESI-HRMS** \(m/z\): [MH]\(^+\) calcd for C\(_{19}\)H\(_{30}\)N\(_5\)O\(_6\)S\(_2\): 488.1637, found: 488.1636.

**HPLC**: retention time: 3.55 min (Chromolith, gradient: 20-50 % B/A over 5 min).

Supplementary figure S31: HPLC trace of crude 5’c.
Arg - 5'm:

ESI-HRMS (m/z): [MH]\(^+\) calcd for C\(_{22}\)H\(_{37}\)N\(_8\)O\(_6\)S\(_2\): 573.2277, found: 573.2272.

HPLC: retention time: 2.54 min (Chromolith, gradient: 20-50 % B/A over 5 min).

Supplementary figure S32: HPLC trace of crude 5'm.
Asn - 5’g:

ESI-HRMS (m/z): [MH]^+ calcd for C_{20}H_{31}N_{6}O_{7}S_{2}: 531.1696, found: 531.1690.

HPLC: retention time: 2.97 min (Chromolith, gradient: 20-50 % B/A over 5 min).

Supplementary figure S33: HPLC trace of crude 5’g.
Asp - 5'b:

**ESI-HRMS** (m/z): [MH]^+ calcld for C_{20}H_{30}N_{5}O_{8}S_{2}: 532.1536, found: 532.1528.

**HPLC**: retention time: 3.48 min (Chromolith, gradient: 20-50 % B/A over 5 min).

---

**Supplementary figure S34**: HPLC trace of crude 5'b.
Cys - 5'e:

**ESI-HRMS** (m/z): [MH]$^+$ calcd for C$_{19}$H$_{30}$N$_5$O$_6$S$_3$: 520.1358, found: 520.1351.

**HPLC**: retention time: 4.07 min (Chromolith, gradient: 20-50 % B/A over 5 min).

In the case of compound 5'e, a minor peak was observed showing the same m/z as expected for compound 5'e. It was attributed to epimerization of Fmoc-Cys(Trt) during coupling, yielding compound S12 after TFA cleavage (ratio: L-Cys / D-Cys 93:7).

**d-Cys - S12:**

**ESI-HRMS** (m/z): [MH]$^+$ calcd for C$_{19}$H$_{30}$N$_5$O$_6$S$_3$: 520.1358, found: 520.1353.

**HPLC**: retention time: 3.41 min (Chromolith, gradient: 20-50 % B/A over 5 min).

Supplementary figure S35: HPLC trace of crude 5'e.
**Gln - 5’j:**

**ESI-HRMS** (m/z): [MH]^+ calcld for C_{21}H_{33}N_{6}O_{7}S_{2}: 545.1852, found: 545.1847.

**HPLC:** retention time: 3.27 min (Chromolith, gradient: 20-50 % B/A over 5 min).

In the case of compound 5’j, concomitant formation of pyroglutamate (S13) was observed during the TFA treatment.

**PyroGlu - S13:**

**ESI-HRMS** (m/z): [MH]^+ calcld for C_{21}H_{30}N_{5}O_{7}S_{2}: 528.1587, found: 528.1579.

**HPLC:** retention time: 4.34 min (Chromolith, gradient: 20-50 % B/A over 5 min).

Supplementary figure S36: HPLC trace of crude 5’j.
Glu 5’d:

**ESI-HRMS** (m/z): [MH]+ calcd for C_{21}H_{32}N_{5}O_{8}S_{2}: 546.1692, found: 546.1684.

**HPLC**: retention time: 3.55 min (Chromolith, gradient: 20-50% B/A over 5 min).

Supplementary figure S37: HPLC trace of crude 5’d.
**Gly - 5’a:**

**ESI-HRMS** (m/z): [MH]+ calcd for C\textsubscript{18}H\textsubscript{28}N\textsubscript{5}O\textsubscript{6}S\textsubscript{2}: 474.1481, found: 474.1476.

**HPLC**: retention time: 3.01 min (Chromolith, gradient: 20-50 % B/A over 5 min).

**Supplementary figure S38**: HPLC trace of crude 5’a.
His - 5’h:

ESI-HRMS (m/z): [MH]⁺ calcd for C_{22}H_{32}N_{7}O_{6}S_{2}: 554.1855, found: 554.1848.

HPLC: retention time: 2.80 min (Chromolith, gradient: 20-50 % B/A over 5 min).

Supplementary figure S39: HPLC trace of crude 5’h.
Ile - 5't:

ESI-HRMS (m/z): [MH]⁺ calcd for C₂₂H₃₆N₅O₆S₂: 530.2107, found: 530.2104.

HPLC: retention time: 4.72 min (Chromolith, gradient: 20-50 % B/A over 5 min).

Supplementary figure S40: HPLC trace of crude 5't.
Fmoc-Ile-OH (0.05 mmol, 10 equiv.) was coupled using a Biotage Initiator+ SP Wave, on 5 µmol of resin 4 using HCTU (19.6 mg, 0.0476 mmol, 9.5 equiv.) and iPr₂NEt (17.4 µL, 0.1 mmol, 20 equiv.) in NMP (0.6 mL) under microwave heating at 70°C for 30 min. After that the peptide resin was treated with 20 % piperidine in NMP (3 × 3 min); finally, resin was cleaved using 1mL of a TFA/H₂O/iPr₃SiH (93:5:2) solution for 2 h. After evaporation of TFA, the sample was analyzed by HPLC; the N-acylation yield was quantified by integration at 315 nm, not taking into account eventual differences in molar extinction coefficient of the products.

**Supplementary figure S41:** HPLC trace of crude 5’t after microwave-assisted coupling.
**Leu - 5′k:**

**ESI-HRMS** (m/z): [MH]$^+$ calcd for $C_{22}H_{36}N_5O_6S_2$: 530.2107, found: 530.2106.

**HPLC:** retention time: 4.66 min (Chromolith, gradient: 20-50 % B/A over 5 min).

**Supplementary figure S42:** HPLC trace of crude 5′k.
Lys - 5’p:

**ESI-HRMS (m/z):** [MH]^+ calcd for C_{22}H_{37}N_{6}O_{6}S_{2}: 545.2216, found: 545.2210.

**HPLC:** retention time: 2.40 min (Chromolith, gradient: 20-50 % B/A over 5 min).

Supplementary figure S43: HPLC trace of crude 5’p.
**Met - 5’f:**

**ESI-HRMS (m/z):** [MH]+ calcd for C_{21}H_{34}N_{5}O_{6}S_{3}: 548.1671, found: 548.1665.

**HPLC:** retention time: 4.56 min (Chromolith, gradient: 20-50 % B/A over 5 min).

---

**Supplementary figure S44:** HPLC trace of crude 5’f.
**Phe - 5'i:**

**ESI-HRMS** \( m/z \): \([MH]^+\) calcd for \( C_{25}H_{34}N_5O_6S_2 \): 564.1950, found: 564.1942.

**HPLC:** retention time: 5.07 min (Chromolith, gradient: 20-50% B/A over 5 min).

**Supplementary figure S45:** HPLC trace of crude 5'i.
**Pro - 5’r:**

**ESI-HRMS** \((m/z)\): \([\text{MH}]^+\) calcd for \(C_{21}H_{32}N_5O_6S_2\): 514.1794, found: 514.1788.

**HPLC:** retention time: 3.86 min (Chromolith, gradient: 20-50 % B/A over 5 min).

---

**Supplementary figure S46:** HPLC trace of crude 5’r.
Ser - 5’o:

ESI-HRMS (m/z): [MH]⁺ calcd for C₁₉H₃₀N₅O₇S₂: 504.1587, found: 504.1580.

HPLC: retention time: 3.13 min (Chromolith, gradient: 20-50 % B/A over 5 min).

Supplementary figure S47: HPLC trace of crude 5’o.
Thr - 5’q:

**ESI-HRMS** (m/z): [MH]$^+$ calcd for C$_{20}$H$_{32}$N$_5$O$_7$S$_2$: 518.1743, found: 518.1736.

**HPLC**: retention time: 3.46 min (Chromolith, gradient: 20-50 % B/A over 5 min).

**Supplementary figure S48**: HPLC trace of crude 5’q.
Trp - 5′I:

ESI-HRMS (m/z): [MH]+ calcd for C_{27}H_{35}N_{6}O_{6}S_{2}: 603.2059, found: 603.2052.

HPLC: retention time: 5.00 min (Chromolith, gradient: 20-50 % B/A over 5 min).

Supplementary figure S49: HPLC trace of crude 5′I.
**Tyr - 5′n:**

**ESI-HRMS (m/z):** [MH]^+ calcd for C_{25}H_{34}N_{5}O_{7}S_{2}: 580.1900, found: 580.1892.

**HPLC:** retention time: 4.18 min (Chromolith, gradient: 20-50 % B/A over 5 min).

Supplementary figure S50: HPLC trace of crude 5′n.
Val - 5's:

ESI-HRMS (m/z): [MH]+ calcd for C_{21}H_{34}N_{5}O_{6}S_{2}: 516.1950, found: 516.1947.

HPLC: retention time: 4.33 min (Chromolith, gradient: 20-50 % B/A over 5 min).

Supplementary figure S51: HPLC trace of crude 5's.
Fmoc-Val-OH (0.05 mmol, 10 equiv.) was coupled using a Biotage Initiator+ SP Wave, on 5 µmol of resin 4 using HCTU (19.6 mg, 0.0476 mmol, 9.5 equiv.) and iPr₂NEt (17.4 µL, 0.1 mmol, 20 equiv.) in NMP (0.6 mL) under microwave heating at 70°C for 30 min. After that the peptide resin was treated with 20 % piperidine in NMP (3 x 3 min); finally, resin was cleaved using 1mL of a TFA/H₂O/iPr₃SiH (93:5:2) solution for 2 h. After evaporation of TFA, the sample was analyzed by HPLC; the N-acylation yield was quantified by integration at 315 nm, not taking into account eventual differences in molar extinction coefficient of the products.

Supplementary figure S52: HPLC trace of crude 5's after microwave-assisted coupling.

9- Kinetics studies of the NCL with model Ac-LYRAA(Hnb)C peptides

9a- Synthesis of the model cysteiny1 peptide 6

Supplementary scheme S16: Synthesis of the model cysteiny1 peptide 6.

Peptide 6 was obtained through automated SPPS (protocol p S3) starting from Fmoc-Rink polystyrene resin (130 mg, 0.79 mmol/g, 0.1 mmol). The peptide-resin
was cleaved (protocol p S3) and 6 was purified by HPLC (Nucleosil, gradient: 32-39 % B/A over 10 min).

6:

**MALDI-TOF** \((m/z)^{+}\): [MH]\(^+\) calcd for C\(_{57}\)H\(_{73}\)N\(_{14}\)O\(_9\)S: 1129.53, found: 1129.53.

**HPLC**: retention time: 26.24 min (Nucleosil, gradient: 5-50 % B/A over 30 min).

Supplementary figure S53: HPLC trace of crude 6.
9b- Influence of the nature of the cysteine C-terminus

Supplementary scheme S17: Native chemical ligations with 7, 8 or 9.

Ligation with 7:

500 μL of a degassed 0.2 M pH 7.1 sodium phosphate buffer containing 25 mM MPAA, 50 mM TCEP and 6 M guanidine hydrochloride were added to 1.2 mg of the peptide 7 (final concentration 2 mM) and 0.7 mg peptide 6 (final concentration 1 mM) under argon. The ligation was carried out at 37°C and monitored by RP-HPLC (Chromolith, gradient: 20-70 % B/A over 6 min). For this, aliquots of 2 μL were diluted in 108 μL of 1.5 % TFA in water and 100 μL were injected in HPLC.

Ligation with 8:

500 μL of a degassed 0.2 M pH 7.1 sodium phosphate buffer containing 25 mM MPAA, 50 mM TCEP and 6 M guanidine hydrochloride were added to 1.05 mg of the
peptide 8 (final concentration 2 mM) and 0.69 mg peptide 6 (final concentration 1 mM) under argon. The ligation was carried out at 37°C and monitored by RP-HPLC (Chromolith, gradient: 20-70 % B/A over 6 min). For this, aliquots of 2 µL were diluted in 108 µL of 1.5 % TFA in water and 100 µL were injected in HPLC.

Ligation with 9:

500 µL of a degassed 0.2 M pH 7.1 sodium phosphate buffer containing 25 mM MPAA, 50 mM TCEP and 6 M guanidine hydrochloride were added to 1.3 mg of the peptide 9 (final concentration 2 mM) and 0.7 mg peptide 6 (final concentration 1 mM) under argon. The ligation was carried out at 37°C and monitored by RP-HPLC (Chromolith, gradient: 20-70 % B/A over 6 min). For this, aliquots of 2 µL were diluted in 108 µL of 1.5 % TFA in water and 100 µL were injected in HPLC.

**Ligation product 14:**

ESI-HRMS (m/z): [MH]+ calcd for C_{86}H_{117}N_{22}O_{16}S: 1745.8739, found: 1745.8773.

HPLC: retention time: 4.46 min (Chromolith, gradient: 20-70 % B/A over 6 min).

Supplementary figure S55: NCL at 15 min using 9.
Supplementary figure S56: NCL after 24h using 9. *: non peptidic compound

Supplementary figure S57: Ligation kinetics observed using 7, 8 or 9.
9c- Study of the hydrolysis of the thioester precursor 9 and epimerization at the ligation site during a typical NCL with 9

In order to quantify the hydrolysis of the thioester and the epimerization at the ligation site during a classical NCL with 6 and 9 (page S60), HPLC standards S14 and 15 were synthesized.

**Supplementary scheme S18**: Hydrolysis of peptide 7 to give HPLC standard S14.⁴

Reaction conditions inspired by Kent et al.⁴

Peptide 7 (1 mM) was incubated under argon in 100 µL of a deoxygenated 200 mM pH 9 sodium phosphate buffer containing 25 mM MPAA, 50 mM TCEP, 200 mM β-mercaptoethanol and 6 M guanidine hydrochloride. The reaction was monitored by RP-HPLC (Chromolith, gradient: 20-70 % B/A over 6 min). After 16h at room temperature, the starting material was consumed and the product S14 was characterized and purified to serve as a HPLC standard.

**S14**:  
MALDI-TOF (m/z): [MH]⁺ calcd for C_{29}H_{47}N_{8}O_{8}: 635.35, found: 635.28.  
HPLC: retention time: 1.04 min (Chromolith, gradient: 20-70 % B/A over 6 min).

⁴ Gates Z. P., Stephan J. R., Lee D. J., Kent S. B. H., *Chem. Comm.*, 2013, 49, 786-788
Supplementary figure S58: HPLC trace of crude S14.

Supplementary scheme S19: Synthesis of HPLC standard 15.

Peptide 15 was obtained through automated SPPS (protocol p S3) starting from Tentagel resin (476 mg, 0.21 mmol/g, 0.1 mmol). The peptide-resin was cleaved (protocol p S3) in order to characterize the corresponding peptide 15.

15:
MALDI-TOF (m/z): [MH]^+ calcd for C_{86}H_{117}N_{22}O_{16}S: 1745.87, found: 1745.84.
HPLC: retention time: 3.94 min (Chromolith, gradient: 20-70 % B/A over 6 min).
Supplementary figure S59: HPLC trace of crude 15.

An HPLC co-injection of the NCL mixture after 24h using 9 with HPLC standards S14 and 15 allowed determining the amount of hydrolysis of the thioester moiety and epimerization at the ligation site after a day of reaction.

| 15 formed (%) | < 0.4 |
|---------------|-------|
| S14 formed (%)| < 4   |

Supplementary table S4: Hydrolysis and epimerization during a typical NCL with 9.

9d- Influence of the buffer

Supplementary scheme S20: NCL in HEPES buffer with 9.

500 µL of a degassed 0.2 M pH 7.1 HEPES buffer containing 25 mM MPAA, 50 mM TCEP and 6 M guanidine hydrochloride were added to 0.8 mg peptide 9 (final concentration 1.5 mM) and 0.7 mg peptide 6 (final concentration 1 mM) under argon.
The ligation was carried out at 37°C and monitored by RP-HPLC (Chromolith, gradient: 20-70 % B/A over 6 min). For this, aliquots of 2 µL were diluted in 108 µL of 1.5 % TFA in water and 100 µL were injected in HPLC.

Supplementary figure S60: Comparison of reaction kinetics using phosphate or HEPES buffer using 9.

9e- Influence of masking phenol or thiol groups

Supplementary scheme S21: Synthesis of O-methylated peptide 12.

Tentagel R resin (476 mg, 0.21 mmol/g, 0.1 mmol) was introduced into a polypropylene syringe fitted with a polypropylene frit and a PTFE stopcock and swollen with NMP. Fmoc group was removed by three successive treatments with 20% piperidine in NMP (10 mL) for 3 min. Then, Fmoc-Rink-OH (270 mg, 0.5 mmol, 5 equiv.) and HATU (190 mg, 0.5 mmol, 5 equiv.) were dissolved in NMP (2 mL) prior to addition of iPr₂NEt (175 µL, 1 mmol, 10 equiv.). The resulting solution was immediately added to the resin. The syringe was left for 1.5 h under gentle stirring,
then the syringe was drained and the resin was washed with NMP. Fmoc group was removed by three successive treatments with 20% piperidine in NMP (10 mL) for 3 min. Fmoc-Gly-OH (297 mg, 1 mmol, 10 equiv.) and HCTU (372 mg, 0.90 mmol, 9 equiv.) were dissolved in NMP (2 mL) prior to addition of iPr₂NEt (348 µL, 2 mmol, 20 equiv.). The resulting solution was immediately added to the resin. The syringe was left for 1 h under gentle stirring, then the syringe was drained and the resin was washed with NMP. Then, Fmoc group was removed by three successive treatments with 20% piperidine in NMP (10 mL) for 3 min. Fmoc-Cys(SBu)-OH (432 mg, 1 mmol, 10 equiv.) and HCTU (372 mg, 0.90 mmol, 9 equiv.) were dissolved in NMP (10 mL) prior to addition of iPr₂NEt (348 µL, 2 mmol, 20 equiv.). The resulting solution was immediately added to the resin. The syringe was left for 1.5 h under gentle stirring, then the syringe was drained and the resin was washed with NMP. Fmoc group was removed by three successive treatments with 20% piperidine in NMP (10 mL) for 3 min. The 2-hydroxy-5-nitrobenzyl linker was then introduced following the general procedure (p S5). The Ac-LYRAA sequence was installed by standard SPPS procedure (protocol p S3), an extended double coupling (2 × 2h) was performed for the first alanine and a normal double coupling (2 × 30 min) was performed for the arginine. Peptide resin was then treated with 20% piperidine in NMP (10 mL, 3 × 3 min), washed with NMP then CH₂Cl₂. Methylation of the phenol group was performed using a solution of MeI (50 equiv.), iPr₂NEt (50 equiv.) in DMF for 4 h and the resin was washed with NMP and CH₂Cl₂. Finally, peptide-resin was cleaved following the general procedure (p S3) to give compound 12, which was purified by semi-preparative HPLC (Nucleosil, gradient: 40-45 % B/A over 10 min).

12:

MALDI-TOF (m/z): [MH]+ calcd for C₄₆H₇₁N₁₂O₁₂S₂: 1047.48, found: 1047.46.
HPLC: retention time: 8.94 min (Chromolith, gradient: 5-50 % B/A over 10 min).
Supplementary figure S61: HPLC trace of crude 12.

Supplementary figure S62: HPLC trace of pure 12.
Supplementary scheme S22: Native chemical ligation with O-methylated 12.

Ligation with 12:

500 µL of a degassed 0.2 M pH 7.1 sodium phosphate buffer containing 25 mM MPAA, 50 mM TCEP and 6 M guanidine hydrochloride were added to 1.05 mg of the peptide 12 (final concentration 2 mM) and 0.69 mg peptide 6 (final concentration 1 mM) under argon. The ligation was carried out at 37°C and monitored by RP-HPLC (Chromolith, gradient: 20-70 % B/A over 6 min). For this, aliquots of 2 µL were diluted in 108 µL of 1.5 % TFA in water and 100 µL were injected in HPLC.

Supplementary scheme S23: Synthesis of the S-Acm peptide 13.

Tentagel R resin (476 mg, 0.21 mmol/g, 0.1 mmol) was introduced into a polypropylene syringe fitted with a polypropylene frit and a PTFE stopcock and swollen with NMP. Fmoc group was removed by three successive treatments with 20% piperidine in NMP (10 mL) for 3 min. Then, Fmoc-Rink-OH (270 mg, 0.5 mmol, 5 equiv.) and HATU (190 mg, 0.5 mmol, 5 equiv.) were dissolved in NMP (2 mL) prior to addition of iPr₂NEt (175 µL, 1 mmol, 10 equiv.). The resulting solution was immediately added to the resin. The syringe was left for 1.5 h under gentle stirring, then the syringe was drained and the resin was washed with NMP. Fmoc group was removed by three successive treatments with 20% piperidine in NMP (10 mL) for 3 min. Fmoc-Gly-OH (297 mg, 1 mmol, 10 equiv.) and HCTU (372 mg, 0.90 mmol, 9 equiv.) were dissolved in NMP (2 mL) prior to addition of iPr₂NEt (348 µL, 2 mmol, 20 equiv.). The resulting solution was immediately added to the resin. The syringe was left for 1 h under gentle stirring, then the syringe was drained and the resin was washed with NMP. Then, Fmoc group was removed by three successive treatments with 20% piperidine in NMP (10 mL) for 3 min. Fmoc-Cys(Acm)-OH (414.5 mg, 1 mmol, 10 equiv.) and HCTU (372 mg, 0.90 mmol, 9 equiv.) were dissolved in NMP (10 mL) prior to addition of iPr₂NEt (348 µL, 2 mmol, 20 equiv.). The resulting
solution was immediately added to the resin. The syringe was left for 1.5 h under gentle stirring, then the syringe was drained and the resin was washed with NMP. Fmoc group was removed by three successive treatments with 20% piperidine in NMP (10 mL) for 3 min. The 2-hydroxy-5-nitrobenzyl linker was then introduced following the general procedure (p S5). The Ac-LYRAA sequence was installed by standard SPPS procedure (protocol p S3), an extended double coupling (2 × 2h) was performed for the first alanine and a normal double coupling (2 × 30 min) was performed for the arginine. Peptide resin was then treated with 20% piperidine in NMP (10 mL, 3 × 3 min), washed with NMP then CH₂Cl₂. Finally, the peptide was cleaved following the general procedure p S3 to give compound 13. 13 has been purified by semi-preparative HPLC (Nucleosil, gradient: 20-35 % B/A over 5 min).

13:
MALDI-TOF (m/z): [MH]+ calcd for C₄₄H₆₆N₁₃O₁₃S: 1016.46, found: 1016.46.
HPLC: retention time: 5.88 min (Chromolith, gradient: 5-50 % B/A over 10 min).

**Supplementary figure S63**: HPLC trace of crude 13.
Ligation with 13:

500 µL of a degassed 0.2 M pH 7.1 sodium phosphate buffer containing 25 mM MPAA, 50 mM TCEP and 6 M guanidine hydrochloride were added to 1.2 mg of the peptide 13 (final concentration 2 mM) and 0.69 mg peptide 6 (final concentration 1 mM) under argon. The ligation was carried out at 37°C and monitored by RP-HPLC (Chromolith, gradient: 20-70 % B/A over 6 min). For this, aliquots of 2 µL were diluted in 108 µL of 1.5 % TFA in water and 100 µL were injected in HPLC.
**Supplementary figure S65:** Ligation kinetics observed using 12 or 13 compared to ligation using 9.

**9f- Influence of the pH**

500 µL of different degassed 0.2 M sodium phosphate buffers (pH 5.6, 6.1, 6.6, 7.1, 7.6) containing 25 mM MPAA, 50 mM TCEP and 6 M guanidine hydrochloride were added to 0.8 mg of the peptide 9 (final concentration 1.5 mM) and 0.69 mg peptide 6 (final concentration 1 mM) under argon. The ligation was carried out at 37°C and monitored by RP-HPLC (Chromolith, gradient: 20-70 % B/A over 6 min). For this, aliquots of 2 µL were diluted in 108 µL of 1.5 % TFA in water and 100 µL were injected in HPLC.

**Supplementary scheme S25:** NCL at different pHs with 9.
Supplementary figure S66: NCL after 24h using 9 at pH 6.6.

Supplementary figure S67: Ligation kinetics observed at different pHs using 9.
10-Influence of the C-terminal aa: NCL with model Ac-LYRAX(Hnb)C peptides

10a- **Synthesis of peptide crypto-thioester Ac-LYRAS-(Hnb)C(S\text{T}Bu)G-NH\textsubscript{2} (10)**

Supplementary scheme S26: Synthesis of model peptide 10.

Peptide 10 was obtained through automated SPPS (protocol p S3) and using the automated reductive amination (protocol p S34) starting from Fmoc-Rink tentagel resin (120 mg, 0.21 mmol/g, 25 µmol). Fmoc-Ser(\text{T}Bu)-OH was coupled for 3 × 30 min on the (Hnb)C(S\text{T}Bu)G-Rink Tentagel resin. The complete sequence was installed through standard Fmoc-SPPS (protocol p S3), Fmoc-Arg(Pbf)-OH was coupled twice (2 × 30 min) and a final piperidine treatment (20 % in NMP, 3 mL, 3 min, ×3) was performed. Peptide-resin was cleaved (protocol p S3) and peptide 10 was purified by semi-preparative RP-HPLC (Nucleosil, gradient: 40-55 % B/A over 12 min).

**10:**

**ESI-HRMS (m/z):** [MH]\textsuperscript{+} calcd for C\textsubscript{45}H\textsubscript{69}N\textsubscript{12}O\textsubscript{13}S\textsubscript{2}: 1049.4548, found: 1049.4532.

**HPLC:** retention time: 2.14 min (Chromolith, gradient: 30-60 % B/A over 5 min).
Supplementary figure S68: HPLC trace of crude 10.

Supplementary figure S69: HPLC trace of pure 10.
10b- NCL with peptide crypto-thioester Ac-LYRAS-(Hnb)C(SfBu)G-NH₂ (10)

Supplementary scheme S27: Native chemical ligation with 10.

250 µL of a degassed 0.2 M pH 7.1 sodium phosphate buffer containing 25 mM MPAA, 50 mM TCEP and 6 M guanidine hydrochloride were added to 0.53 mg of the peptide 10 (final concentration 2 mM) and 0.30 mg peptide 6 (final concentration 1 mM) under argon. The ligation was carried out at 37°C and monitored by RP-HPLC (Chromolith, gradient: 20-70 % B/A over 6 min). For this, aliquots of 2 µL were diluted in 108 µL of 1.5 % TFA in water and 100 µL were injected in HPLC.

Ligation product 16:

ESI-HRMS (m/z): [MH]+ calcd for C₈₆H₁₁₇N₂₂O₁₇S: 1761.8688, found: 1761.8687.
HPLC: retention time: 3.81 min (Chromolith, gradient: 20-70 % B/A over 6 min).

Supplementary figure S70: NCL after 25h using 10.
Peptide 11 was obtained through automated SPPS (protocol p S3) and using the automated reductive amination (protocol p S34) starting from Fmoc-Rink tentagel resin (120 mg, 0.21 mmol/g, 25 µmol). Fmoc-Val-OH has been coupled for 5 × 30 min on the (Hnb)C(SfBu)G-Rink Tentagel resin. The complete sequence was installed through standard Fmoc-SPPS (protocol p S3), Fmoc-Arg(Pbf)-OH was coupled for 2 × 30 min and a final piperidine (20 % in NMP, 3mL, 3 min, ×3) was performed. Peptide-resin was cleaved (protocol p S3) and peptide 11 was purified by semi-preparative RP-HPLC (Nucleosil, gradient: 40-55 % B/A over 12 min).

**11:**

**ESI-HRMS (m/z):** [MH]$^+$ calcd for C$_{47}$H$_{73}$N$_{12}$O$_{12}$S$_2$: 1061.4912, found: 1061.4891.

**HPLC:** retention time: 3.51 min (Chromolith, gradient: 30-60 % B/A over 5 min).

*: minor compound observed (Val deletion) resulting from incomplete coupling of Fmoc-Val-OH because no acetylation-mediated capping step was performed.

**:*:

**ESI-HRMS (m/z):** [MH]$^+$ calcd for C$_{42}$H$_{64}$N$_{11}$O$_{11}$S$_2$: 962.4228, found: 962.4224.

**HPLC:** retention time: 3.51 min (Chromolith, gradient: 30-60 % B/A over 5 min).
Supplementary figure S71: HPLC trace of crude 11.

Supplementary figure S72: HPLC trace of pure 11.
10d- NCL with peptide crypto-thioester Ac-LYRAV-(Hnb)C(S\text{StBu})G-NH$_2$ (11) under optimized conditions

Supplementary scheme S29: Native chemical ligation with 11.

250 µL of a degassed 0.2 M pH 7.1 sodium phosphate buffer containing 25 mM MPAA, 50 mM TCEP and 6 M guanidine hydrochloride were added to 0.53 mg of the peptide 11 (final concentration 2 mM) and 0.30 mg peptide 6 (final concentration 1 mM) under argon. The ligation was carried out at 37°C and monitored by RP-HPLC (Chromolith, gradient: 20-70 % B/A over 6 min). For this, aliquots of 2 µL were diluted in 108 µL of 1.5 % TFA in water and 100 µL were injected in HPLC.

10e- NCL with peptide crypto-thioester Ac-LYRAV-(Hnb)C(S\text{StBu})G-NH$_2$ (11) under optimized conditions

Supplementary scheme S30: Native chemical ligation with 11.

500 µL of a degassed 0.2 M pH 6.6 sodium phosphate buffer containing 300 mM MPAA, 50 mM TCEP and 6 M guanidine hydrochloride were added to 1.2 mg of the peptide 11 (final concentration 2 mM) and 0.68 mg peptide 6 (final concentration 1 mM) under argon. The ligation was carried out at 50°C and monitored by RP-HPLC (Chromolith, gradient: 20-70 % B/A over 6 min). For this, aliquots of 2 µL were diluted in 108 µL of 1.5 % TFA in water, this aqueous phase was washed with di-ethyl ether (500 µL ×3) and 100 µL were injected in HPLC.

**Ligation product 17:**

ESI-HRMS ($m/z$): [MH]$^+$ calcd for C$_{88}$H$_{121}$N$_{22}$O$_{16}$S: 1773.9052, found: 1773.9059.

HPLC: retention time: 5.0 min (Chromolith, gradient: 20-70 % B/A over 6 min).
**Supplementary figure S73:** NCL after 24h using 11 under optimized conditions. *:* non peptidic compound

---

10f- **Determination of apparent second order kinetic constants**

Kinetics of selected representative NCL reactions were modeled as an apparent second order reaction, considering the chemical equation:

\[
\text{H-CysPeptide}_1 + \text{Peptide}_2-(\text{Hnb})\text{Cys} \xrightarrow{k_{\text{app}}} \text{Peptide}_2\text{CysPeptide}_1
\]

following the general rate equation for \([A]_0 \neq [B]_0\):

\[
k_t = \frac{1}{[B]_0'[A]_0} \ln \left( \frac{[A]_0 - ([B]_0'[A])}{[B]_0 - ([A]_0'[A])} \right)
\]

Values for \(t < 10\text{min}\) were not taken into account due to the early SfBu deprotection step.
Supplementary figure S74: Apparent second order kinetic constant for NCL using 9. (Conditions corresponding to Figure 3 of the article)
Supplementary figure S75: Apparent second order kinetic constant for NCL using 12.
(Conditions corresponding to Figure 3 of the article)
Supplementary figure S76: Apparent second order kinetic constant for NCL using 10. (Conditions corresponding to Figure 3 of the article)
Supplementary figure S77: Apparent second order kinetic constant for NCL using 11 (Conditions corresponding to Figure 3 of the article)

11-Application to MT7

11a- Synthesis of cysteiny1 peptide MT7 [42-65] (19)

Amino acid sequence of the MT7 [42-65] segment:

H-CAATCPKAERYRDVINCCGTDKCNK-OH

Supplementary scheme S31: Synthesis of cysteiny1 peptide MT7 [42-65] 19.

Peptide 19 was obtained through automated SPPS (protocol p S3) starting from Tentagel resin (476, 0.21 mmol/g, 0.1 mmol). Fmoc-K(Boc)-Mppa-OH (323.3 mg, 0.5 mmol, 5 equiv.) and HATU (190 mg, 0.5 mmol, 5 equiv.) were dissolved in NMP (2 mL) prior to addition of iPr₂NEt (175 µL, 1 mmol, 10 equiv.). The resulting solution was immediately added to the resin. The syringe was left for 1.5 h under gentle stirring, then the syringe was drained and the resin was washed with NMP. Fmoc
group was removed by three successive treatments with 20% piperidine in NMP (10 mL) for 3 min and the complete sequence was installed through standard Fmoc-SPPS (elongation yield = 71 %). After cleavage of a quarter of the resin (25 µmol, protocol p S3), product 19 was purified by semi-preparative RP-HPLC (Nucleosil, gradient: 15-23 % B/A over 16 min), 4.06 µmol were obtained (yield = 16.2 %, UV titration at 280 nm, molar extinction coefficient = 1290 L.mol⁻¹.cm⁻¹).

19:

**ESI-HRMS** (m/z): [MH]⁺ calcd for C₁₀₄H₁₇₃N₃₂O₃₆S₅: 2606.1293, found: 2606.1277.

**HPLC**: retention time: 3.33 min (Chromolith, gradient: 10-40 % B/A over 5 min).

Supplementary figure S78: HPLC trace of crude 19.

Supplementary figure S79: LC/MS analysis of crude 19.
| Peak (tr (min)) | [MH]$^+$ (m/z) | [MH]$^+$ (m/z) found | Attributed to |
|----------------|----------------|----------------------|--------------|
| 7 (4.11)       | 1013.3855      | 1013.3823            | Ac-[57-65]   |
| 18 (6.96)      | 1240.5125      | 1240.5095            | Ac-[55-65]   |
| 24 (7.67)      | 1610.7089      | 1610.7057            | Ac-[52-65]   |
| 28 (8.09)      | 1339.5809      | 1339.5755            | Ac-[54-65]   |
| 32 (8.38)      | 2606.1293      | 2606.1277            | 19           |
| 33 (8.51)      | -              | 2588.1143            | Not attributed |
| 35 (8.83)      | 2198.9997      | 2198.9964            | Ac-[47-65]   |
| 38 (9.20)      | -              | 1886.7800            | Not attributed |
| 39 (9.34)      | 2662.1919      | 2662.1880            | 19 + 56 Da (tBu) |
| 41 (9.41)      | 2662.1919      | 2662.1884            | 19 + 56 Da (tBu) |
| 43 (9.79)      | 2662.1919      | 2662.1869            | 19 + 56 Da (tBu) |
| 44 (9.91)      | 2662.1919      | 2662.1872            | 19 + 56 Da (tBu) |

**Supplementary table S5:** Attribution of selected peaks observed in LC/MS analysis of crude 19.

**Absorbance (λ = 214 nm) →**

**Supplementary figure S80:** HPLC trace of pure 19.
Tentagel R resin (476 mg, 0.21 mmol/g, 0.1 mmol) was introduced into a polypropylene syringe fitted with a polypropylene frit and a PTFE stopcock and swollen with NMP. Fmoc group was removed by three successive treatments with 20% piperidine in NMP (10 mL) for 3 min. Then, Fmoc-Rink-OH (270 mg, 0.5 mmol, 5 equiv.) and HATU (190 mg, 0.5 mmol, 5 equiv.) were dissolved in NMP (2 mL) prior to addition of iPr2NEt (175 µL, 1 mmol, 10 equiv.). The resulting solution was immediately added to the resin. The syringe was left for 1.5 h under gentle stirring, then the syringe was drained and the resin was washed with NMP. Fmoc group was removed by three successive treatments with 20% piperidine in NMP (10 mL) for 3 min. Fmoc-Gly-OH (297 mg, 1 mmol, 10 equiv.) and HCTU (372 mg, 0.90 mmol, 9 equiv.) were dissolved in NMP (2 mL) prior to addition of iPr2NEt (348 µL, 2 mmol, 20 equiv.). The resulting solution was immediately added to the resin. The syringe was left for 1 h under gentle stirring, then the syringe was drained and the resin was washed with NMP. Then, Fmoc group was removed by three successive treatments with 20% piperidine in NMP (10 mL) for 3 min. Fmoc-Cys(SbBu)-OH (432 mg, 1 mmol, 10 equiv.) and HCTU (372 mg, 0.90 mmol, 9 equiv.) were dissolved in NMP (10 mL) prior to addition of iPr2NEt (348 µL, 2 mmol, 20 equiv.). The resulting solution was immediately added to the resin. The syringe was left for 1.5 h under gentle stirring, then the syringe was drained and the resin was washed with NMP. Then, Fmoc group was removed by three successive treatments with 20% piperidine in NMP (10 mL) for 3 min. The N-2-hydroxy-5-nitrobenzyl group was then introduced following the general procedure (p S5). Then, the complete MT7 [1-41] sequence was installed on a quarter of the resin through standard Fmoc-SPPS (25 µmol, protocol S3). After cleavage of the resin (protocol p S3), the crude product 18 was pre-purified using a Sep-Pak cartridge (Waters Vac 12cc, C18 - 2g, washing: 50 mL 0 % B/A then 50 mL 20 % B/A and elution: 50 mL 40 % B/A) and then purified by semi-preparative RP-HPLC (Nucleosil, gradient: 35-43 % B/A over 8 min) 1.62 µmol were
obtained (yield of 12.96 %, UV titration at 280 nm, molar extinction coefficient = 15940 L.mol⁻¹.cm⁻¹).

18:

ESI-HRMS (m/z): [MH]⁺ calcd for C_{234}H_{345}N_{62}O_{67}S_{6}: 5287.3819, found: 5287.3818.

HPLC: retention time: 2.82 min (Chromolith, gradient: 30-50 % B/A over 5 min).

Supplementary figure S81: HPLC trace of crude 18.

Supplementary figure S82: LC/MS analysis of crude 18.
| Peak (tr (min)) | [MH]$^+$ (m/z) Calc. | [MH]$^+$ (m/z) found | Attributed to |
|----------------|----------------------|----------------------|--------------|
| 1 (11.53)      | -                    | 1826.8617            | Not attributed |
| 2 (12.87)      | -                    | 1886.9243            | Not attributed |
| 3 (14.06)      | -                    | 614.2409             | Not attributed |
| 4 (14.72)      | -                    | 3479.5302            | Not attributed |
| 5 (14.86)      | 5303.3768            | 5303.3519            | 18 +16 Da (Oxidized Met) |
| 10 (15.14)     | 2691.2423            | 2691.2184            | Ac-[25-41]-HnbC(SiBu)-G-NH$_2$ |
| 12 (15.31)     | 5287.3819            | 5287.3818            | 18 |
| 13 (15.45)     | -                    | 5287.3423            | Not attributed |
| 14 (15.48)     | -                    | 5290.3463            | Not attributed |
| 16 (15.80)     | 5343.4444            | 5343.4466            | 18 + 56 Da (tBu) |

Supplementary table S6: Attribution of selected peaks observed in LC/MS analysis of crude 18.

Supplementary figure S83: HPLC trace of pure 18.
170 µL of a degassed 0.2 M pH 6.3 sodium phosphate buffer containing 25 mM MPAA, 50 mM TCEP and 6 M guanidine hydrochloride were added to 1.05 mg of the MT7 [1-41]-HhbC(S/tBu)G-NH₂ peptide 18 (0.168 µmol, final concentration 1 mM) and 0.66 mg of the MT7 [42-65] peptide 19 (0.218 µmol, 1.3 equiv., final concentration 1.33 mM) under argon. The ligation was carried out at 37°C and monitored by RP-HPLC (Chromolith, gradient: 10-60% B/A over 6 min). For this, aliquots of 2 µL were diluted in 108 µL of 1.5% TFA in water and 100 µL were injected to the HPLC. After 24h, the reaction mixture was acidified with TFA (10 µL), 1 mL solvent A and 200 µL solvent B. this solution was extracted with diethylether (4 x 10 mL) to remove the MPAA. TCEP was added (final 100 mM) and the pH was adjusted to 5.0; after 20 min the pH was adjusted to 1 and the ligation product 20 was purified by semi-preparative RP-HPLC (Nucleosil, gradient: 25-43 % B/A over 10 min). 23.4 nmol were obtained (yield = 14 %, UV titration at 280 nm, molar extinction coefficient = 14870 L.mol⁻¹.cm⁻¹).

20:

ESI-HRMS (m/z): [MH]⁺ calcd for C₃₂₂H₄₉₃N₈₀O₉₈S₉: 7476.3847, found: 7476.4022.

HPLC: retention time: 4.34min (Chromolith, gradient: 10-60% B/A over 6 min).
Supplementary figure S84: NCL of MT7 [1-65] 20. *: MPAA

Supplementary figure S85: HPLC trace of pure MT7 [1-65] 20.
12-Application to Cg-BigDef1

12a- Synthesis of cysteinyl peptide Cg-BigDef1 [57-93] (22)

Amino acid sequence of the Cg-BigDef1 [57-93] segment:

H-CANNRGWCRTCFSHEYTDWFNNVCGSYRCCRPGRR-NH₂

Supplementary scheme S34: Synthesis of cysteinyl peptide Cg-BigDef1 [57-93] peptide 22.

Peptide 22 was obtained through automated SPPS (protocol p S3) starting from H-Rink amide Chemmatrix resin (200 mg, 0.52 mmol/g, 0.1 mmol); the elongation yield was 64% (determined by UV titration). After cleavage of a part of the resin (21 µmol) (protocol p S3), the product 22 was purified by semi-preparative RP-HPLC (Nucleosil, gradient: 25-38 % B/A over 13 min) 3.03 µmol were obtained (yield = 14.4%, UV titration at 280 nm, molar extinction coefficient = 13580 L.mol⁻¹.cm⁻¹).

22:

ESi-HRMS (m/z): [MH]+ calcd for C₁₈₅H₂₇₁N₆₄O₆₃S₆: 4428.8802, found: 4428.8855.

HPLC: retention time: 3.57 min (Chromolith, gradient: 23-34 % B/A over 5 min).

Supplementary figure S86: HPLC trace of crude 22.
Supplementary figure S87: LC/MS analysis of crude 22.

| Peak (tr (min)) | [MH]^+ (m/z) | [MH]^+ (m/z) found | Attributed to          |
|----------------|--------------|---------------------|-----------------------|
| 1 (1.79)       | 888.4409     | 888.4405            | Ac-[87-93]            |
| 2 (1.82)       | 888.4409     | 888.4401            | Ac-[87-93]            |
| 3 (2.79)       | 1351.6588    | 1351.6580           | Ac-[83-93]            |
| 4 (3.22)       | 1454.6680    | 1454.6670           | Ac-[82-93]            |
| 7 (3.75)       | 1896.8492    | 1896.8480           | Ac-[78-93]            |
| 8 (3.81)       | 1782.8063    | 1782.8043           | Ac-[79-93]            |
| 9 (3.90)       | 1668.7634    | 1668.7612           | Ac-[80-93]            |
| 16 (6.14)      | 2229.9969    | 2229.9911           | Ac-[76-93]            |
| 20 (6.46)      | 4428.8802    | 4428.8855           | 22                     |
| 23 (6.72)      | 2738.1775    | 2738.1721           | Ac-[72-93]            |
| 26 (6.99)      | 4484.9481    | 4484.9377           | 22 + 56 Da (tBu)      |
| 28 (7.36)      | 3410.4465    | 3410.4426           | Ac-[66-93]            |

Supplementary table S7: Attribution of selected peaks observed in LC/MS analysis of crude 22.
Supplementary figure S88: HPLC trace of pure 22.

12b- Synthesis of crypto-thioester peptide Cg-BigDef1 [1-56]-(Hnb)C(S\text{Bu})G-NH\text{2} (21)

Amino acid sequence of the Cg-BigDef1 [1-56]-(Hnb)C(S\text{Bu})G-NH\text{2} segment:
XAQALLPIASYAGLTVSAPVFAALVTVYGAYALYRYNIRRRENSYQRIRSDHS(Hnb)C(S\text{Bu})G-NH\text{2}
X = pyroglutamic acid

Supplementary scheme S35: Synthesis of crypto-thioester peptide Cg-BigDef1 [1-56]-(Hnb)C(S\text{Bu})G-NH\text{2} peptide 21.

Peptide 21 was obtained through automated SPPS (protocol p S3) and using the automated reductive amination (protocol p S34) starting from Fmoc-Rink tentagel resin (120 mg, 0.21 mmol/g, 25 µmol) and for some amino acids double or triple coupling have been applied (see below) as determined as difficult couplings from a previous synthesis of the sequence not incorporating an (Hnb)Cys transthioesterification device. After cleavage of the resin (protocol p S3), the product 21 was purified by semi-preparative RP-HPLC (Nucleosil, gradient: 48-58 % B/A over
30 min) 1.69 µmol were obtained (yield = 6.8 %, UV titration at 280 nm, molar extinction coefficient = 10100 L.mol⁻¹.cm⁻¹).

XAQALLPIASYAGLTVSAPVFAALVTYYGAYALRYRNIRRENYQRIRSDHDSHS(Hnb)C(StBu)G-NH₂
X = pyroglutamic acid; green = double coupling, blue = triple coupling

21:

ESI-HRMS (m/z): [MH]^+ calcd for C₂₉₈H₄₅₇N₈₆O₈₆S₂: 6680.3472, found: 6680.3554
HPLC: retention time: 5.40 min (Chromolith, gradient: 20-70 % B/A over 6 min).

Supplementary figure S89: HPLC trace of crude 21.

Supplementary figure S90: LC/MS analysis of crude 21.
| Peak (tr (min)) | [MH]^+ (m/z)         | [MH]^+ (m/z) found | Attributed to                          |
|----------------|-----------------------|-------------------|----------------------------------------|
| 4 (9.16)       | 2966.3611             | 2966.3649         | Ac-[37-56]-(Hnb)C(SfBu)-G-NH₂          |
| 13 (10.21)     | -                     | 3545.6465         | Not attributed                         |
| 15 (10.65)     | 3923.8319             | 3923.8386         | Ac-[29-56]-(Hnb)C(SfBu)-G-NH₂          |
| 23 (11.59)     | 1493.5902             | 1493.5893         | Ac-[48-56]-(Hnb)C(SfBu)-G-NH₂          |
| 46 (17.61)     | 5583.7555             | 5583.7657         | Ac-[12-56]-(Hnb)C(SfBu)-G-NH₂          |
| 47 (17.75)     | 5904.8880             | 5904.8903         | Ac-[9-56]-(Hnb)C(SfBu)-G-NH₂           |
| 48 (17.83)     | 5746.8189             | 5746.8267         | Ac-[11-56]-(Hnb)C(SfBu)-G-NH₂          |
| 49 (18.24)     | 4984.4277             | 4984.4364         | Ac-[19-56]-(Hnb)C(SfBu)-G-NH₂          |
| 51 (18.97)     | 6115.0248             | 6115.0354         | Ac-[7-56]-(Hnb)C(SfBu)-G-NH₂           |
| 55 (20.20)     | 6680.3472             | 6680.3554         | 21                                      |
| 60 (21.88)     | -                     | 6776.3467         | Not attributed                         |

**Supplementary table S8**: Attribution of selected peaks observed in LC/MS analysis of crude 21.

**Supplementary figure S91**: HPLC trace of pure 21.
352.5 µL of a degassed 0.2 M pH 6.3 sodium phosphate buffer containing 50 mM MPAA, 100 mM TCEP and 6 M guanidine hydrochloride were added to 5.38 mg of the Cg-BigDef1 [1-56]-(Hnb)C(SBu)G-NH₂ peptide 21 (0.705 µmol, final concentration 2 mM) and 4 mg of the Cg-BigDef1 [57-93] peptide 22 (0.763 µmol, 1.08 equiv., final concentration 2.16 mM) under argon. The ligation was carried out at 37°C and monitored by RP-HPLC (Chromolith, gradient: 20-70 % B/A over 6 min). For this, aliquots of 2 µL were diluted in 108 µL of 1.5% TFA in water and 100 µL were injected to the HPLC. After 24h, the reaction mixture was acidified with TFA (30 µL) and 2 mL of 1.5% TFA in water; this solution was extracted with diethylether (4 × 10 mL) to remove the MPAA. The ligation product precipitated; after centrifugation and removal of the supernatant, the solid was diluted in 400 µL of solvent B and 1.2 mL of solvent A (V total = 1.6 mL); 45 mg of TCEP were added (final 100 mM) and the pH was adjusted to 4.5, after 20 min the pH was adjusted to 1 and the ligation product SXX was purified by semi-preparative RP-HPLC (Nucleosil, gradient: 50-60 % B/A over 10 min). 130 nmol were obtained (yield = 18.4 %, UV titration at 280 nm, molar extinction coefficient = 21320 L.mol⁻¹.cm⁻¹).

23:

ESI-HRMS (m/z): [MH]+ average mass calcd for C₄₆₇H₇₀₃N₁₄₆O₁₃₄S₆ : 10699.0090, found: 10699.1541

HPLC: retention time: 4.96 min (Chromolith, gradient: 20-70 % B/A over 6 min).
Supplementary figure S92: NCL reaction to obtain Cg-BigDef1 [1-93] 23. *: MPAA
Supplementary figure S93: HPLC trace of pure Cg-BigDef1 [1-93] 23.