Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Efficient synthesis of cysteine-rich cyclic peptides through intramolecular native chemical ligation of *N*-Hnb-Cys peptide crypto-thioesters

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Supporting Information

Table of contents:

1) General information S2
2) General procedures for solid phase peptide synthesis S3
3) Syntheses of peptide linear precursors **1a-5a** S3
4) Peptides cyclization *via* intramolecular NCL S17

1. General information

All reagents and solvents were used without further purification. Protected amino acids, Fmoc-Rink linker, HCTU and HATU were purchased from Merck Biosciences (Nottingham, UK). Aminomethyl TentaGel R resin was purchased from Rapp polymers (Tuebingen, Germany). Peptide synthesis grade DMF was purchased from VWR (Fontenay-sous-Bois, 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).

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. The multiply-charged envelope was deconvoluted using the Charge Deconvolution algorithm in Bruker Data Analysis 4.1 software to obtain the monoisotopic $[M+H]^+$ molecular ion value.

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, 100 × 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. Chromatography was conducted at room temperature unless otherwise mentioned. Solvents A and B are 0.1% TFA in H_2O and 0.1% TFA in MeCN, respectively. Each gradient was followed by a washing step (95% B/A for 0.5 min for Chromolith; for 1 min for Nucleosil) to identify eventual co-products not eluted during the gradient. LC/HRMS analyses were carried out on an Ultimate® 3000 RSLC 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, 0.5 mL/min flow rate, 40°C) column. Solvents A and B were 0.1% formic acid in H_2O and 0.08% formic acid in MeCN, respectively. Gradient: 3% B for 0.6 min, then 3 to 50% B over 10.8 min.

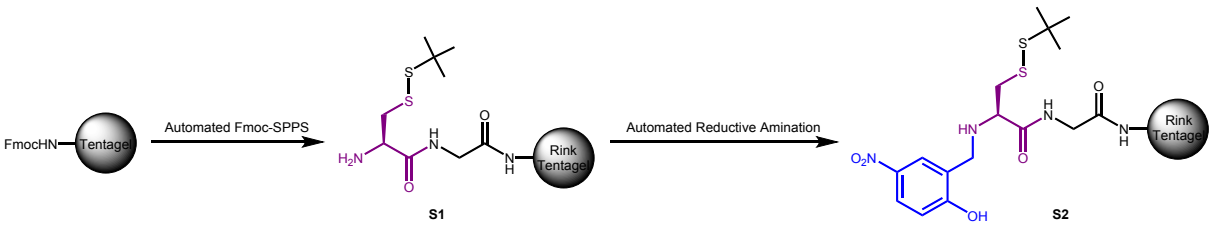
Yields of linear crypto thioesters **1a**-**5a** were calculated from the initial resin loading, by evaluating the quantities of purified peptides by weight, taking into account a molecular mass including trifluoroacetate counter-ions (one per Arg, His, Lys and N-terminal amine of the peptide sequence) but not eventual hydration. Ligation yields were determined by UV spectrophotometry at 280 nm in 8:2:0.01 H_2O/MeCN/TFA (ε^{280}(Hnb) = 3440 $L.mol^{-1}.cm^{-1}$, (ε^{280}(Trp) = 5500 $L.mol^{-1}.cm^{-1}$ and (ε^{280}(Tyr) = 1290 $L.mol^{-1}.cm^{-1}$) except for **4b** and **5b** that do not contain tryptophan or tyrosine (yields evaluated by weight).

2. General procedures for solid phase peptide synthesis

Fmoc-based solid phase peptide syntheses (SPPS) were carried out on a Prelude synthesizer from Protein Technologies (Tucson, Arizona USA). Standard side-chain protecting groups were used: Arg(Pbf), Asn(Trt), Asp(O*t*Bu), Cys(Trt), Glu(O*t*Bu), Gln(Trt), His(Trt), Lys(Boc), Ser(*t*Bu), Thr(*t*Bu), Trp(Boc) and Tyr(*t*Bu), as well as Cys(S*t*Bu) for the thioesterification device.
Syntheses were performed at a 25 µmol scale. Protected amino acids (0.25 mmol, 10 equiv.) were coupled using HCTU (98 mg, 0.238 mmol, 9.5 equiv.) and iPr$_2$NEt (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.), iPr$_2$NEt (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 removed by three successive treatments with 20% piperidine in NMP (3 mL) for 3 min.

The crude peptides were deprotected and cleaved from the resin through a treatment with TFA/H$_2$O/iPr$_3$SiH/phenol (88:5:2:5) for 2 h, then precipitated by dilution into an ice-cold 1:1 diethyl ether/petroleum ether mixture, recovered by centrifugation, further washed three times with diethyl ether and dried under reduced pressure..

3. Syntheses of peptide linear precursors 1a-5a



Supplementary scheme S1: Synthesis of peptide-resin **S2**.

Rink linker, Gly and Cys(S*t*Bu) were successively coupled by automated SPPS on a Tentagel R resin (120 mg, 0.21 mmol/g, 25 µmol) in order to obtain peptide-resin **S1**.

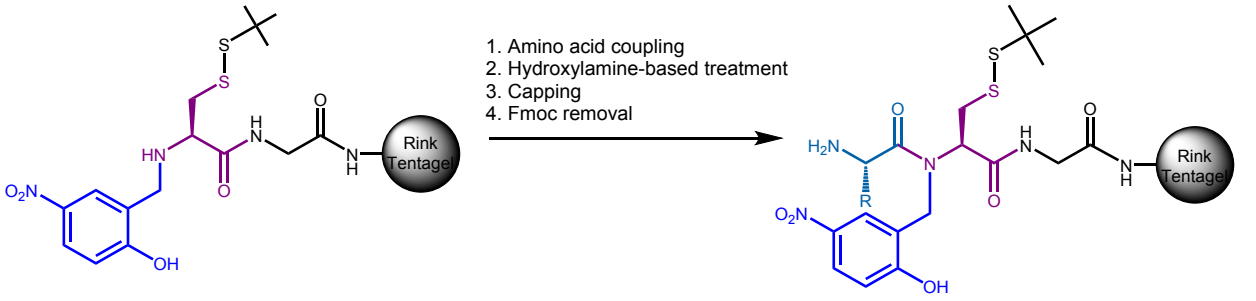
Reductive amination:

Peptide-resin **S1** (25 µmol) was washed two times with 3 mL of a 1:1 DMF/MeOH mixture for 30 s, then swollen in 3 mL of a 9:9:2 DMF/MeOH/AcOH mixture for 5 min. The reactor was drained off and the resin was washed four times with 3 mL of a 1:1 DMF/MeOH mixture for 30 s. This process forms the acetic acid salt of the amine group of cysteine.

2-Hydroxy-5-nitrobenzaldehyde (HNBA) in 1:1 DMF/MeOH (125 mM, 10 equiv., 2 mL) was then added and the reactor was left for 1 h under stirring through nitrogen

bubbling. The reactor was drained and the resin was washed four times with 3 mL of 1:1 DMF/MeOH for 15 s.

Without delay, a fresh solution of sodium cyanoborohydride in 9:9:2 DMF/MeOH/AcOH (250 mM, 20 equiv. 2 mL) were added and the reactor was left for 1 h under stirring by nitrogen bubbling. The reactor was drained off and the resin was washed with 1:1 DMF/MeOH (3 mL, 30 s, × 4), NMP (3 mL, 30 s, × 3), 20% piperidine in NMP (3 mL, 30 s, × 3), NMP (3 mL, 30 s, × 3), dichloromethane (5 mL, 30 s, × 3) and NMP (3 mL, 30 s, × 2).



Supplementary scheme S2: Installation of the C-terminal amino acid of the sequence and determination of the *N*-acylation yield.

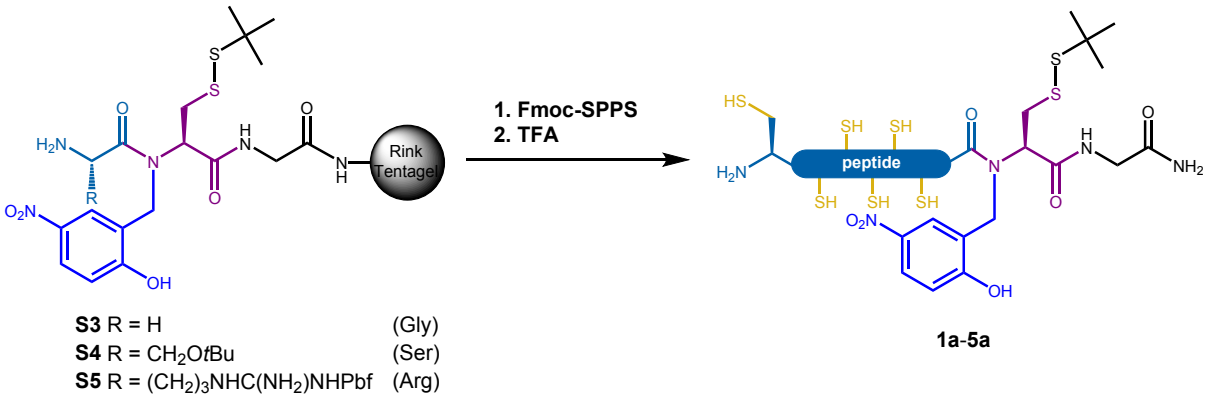
The C-terminal amino acid of the target sequence was coupled twice (except for Fmoc-Ser(*t*Bu)-OH: three times) on peptide-resin **S2** (general procedure p S3). Peptide-resin was then treated with a solution of hydroxylamine hydrochloride (0.3 M) and imidazole (0.225 M) in 5:1 NMP/CH_2Cl_2 (3 mL, 20 min, × 3). After a capping step (general procedure p S3), the Fmoc group was removed through a standard piperidine treatment (p S3) and the *N*-acylation yield was determined by UV spectrophotometry at 301 nm (fluorenylmethylpiperidine byproduct: ε= 7800 $L \cdot mol^{-1} \cdot cm^{-1}$) (see table S1).

Peptide sequence	Cycloviolacin O2	Kalata B1	Cter M	RTD-1	SFTI-1
Peptide-resin	**S3**		**S4**	**S5**	
Amino acid	Gly		Ser(*t*Bu)	Arg(Pbf)	
N-acylation yield (%)	89		85	77	

Supplementary table S1: *N*-acylation yield for the introduction of the C-terminal amino acid of the sequence.

Peptide elongation:

		peptide sequence	lenght (aa)	number of Cys
Cycloviolacin O2	**1:**	CSCKSKVCYRNGIPCGESCVWIPCISSAIG	30	6
Cter M	**2:**	CSWPICMKNGLPTCGETCTLGTCYVPDCS	29	6
Kalata B1	**3:**	CTCSWPVCTRNGLPVCGETCVGGTCNTPG	29	6
RTD-1	**4:**	CICTRGFCRCLCRRGVCR	18	6
SFTI-1	**5:**	CTKSIPPICFPDGR	14	2



S3 R = H (Gly)
S4 R = CH_2O*t*Bu (Ser)
S5 R = $(CH_2)_3NHC(NH_2)NHPbf$ (Arg)

Supplementary scheme S3: Fmoc-based SPPS elongation of crypto-thioester peptides **1a**-**5a**.

Then, the five different peptide sequences were elongated through standard Fmoc-based SPPS (general procedure p S3). Elongation yields were determined by UV spectrometry (deprotection of the Fmoc group of the first and last amino acid residues of the sequence). For this purpose, after the coupling of the N-terminal cysteine, peptide-resins were treated with a solution of hydroxylamine hydrochloride (0.3 M) and imidazole (0.225 M) in 5:1 NMP/CH_2Cl_2 (3 mL, 20 min, × 3) prior to the piperidine treatment, in order to cleave any Fmoc-Cys(Trt) ester on the Hnb moiety. A final TFA treatment (general procedure p S3) afforded peptides **1a**-**5a** that were purified by RP-HPLC.

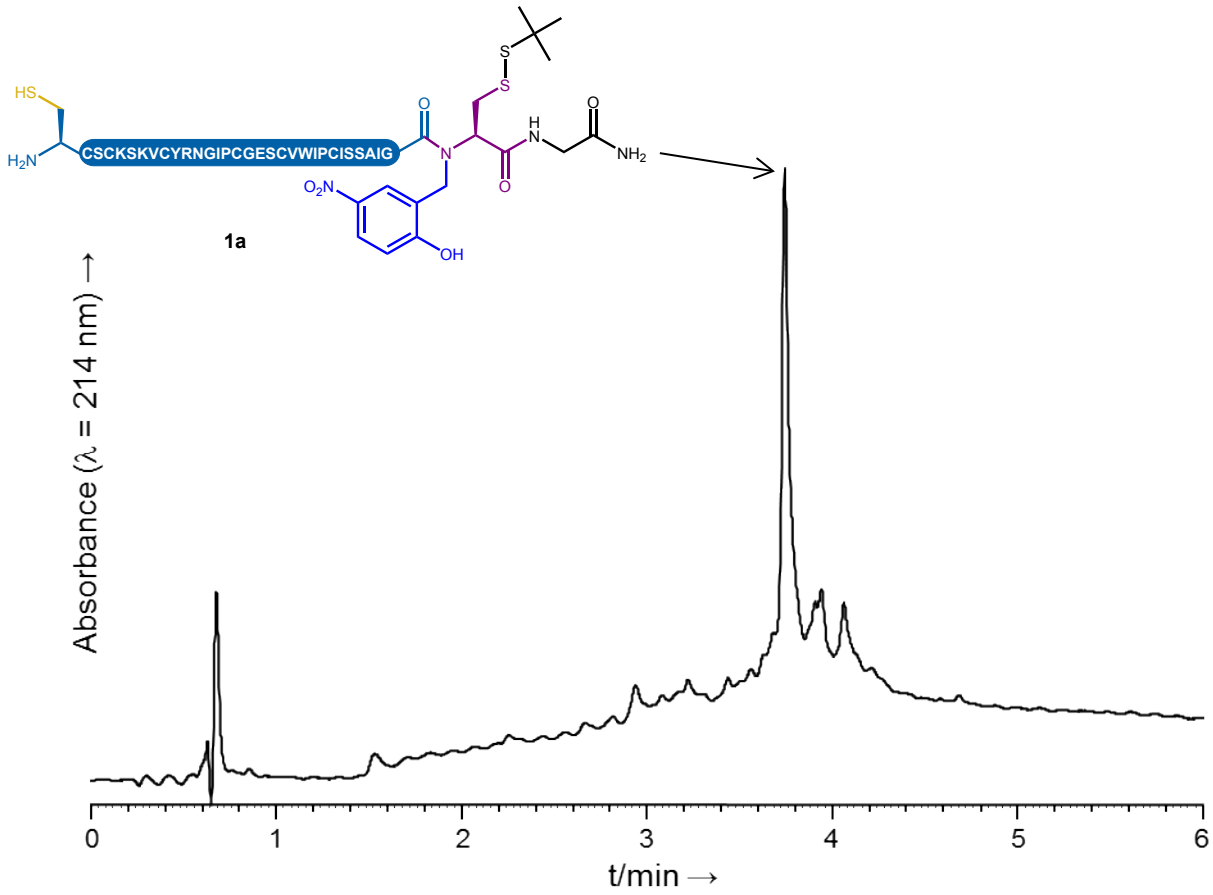
Peptide	**1a**	**2a**	**3a**	**4a**	**5a**
Elongation yield (%)	56	65	56	74	85
Isolated yield (%)	18	10	16	11	21

Supplementary table S2: Yields for peptides **1a**-**5a**.

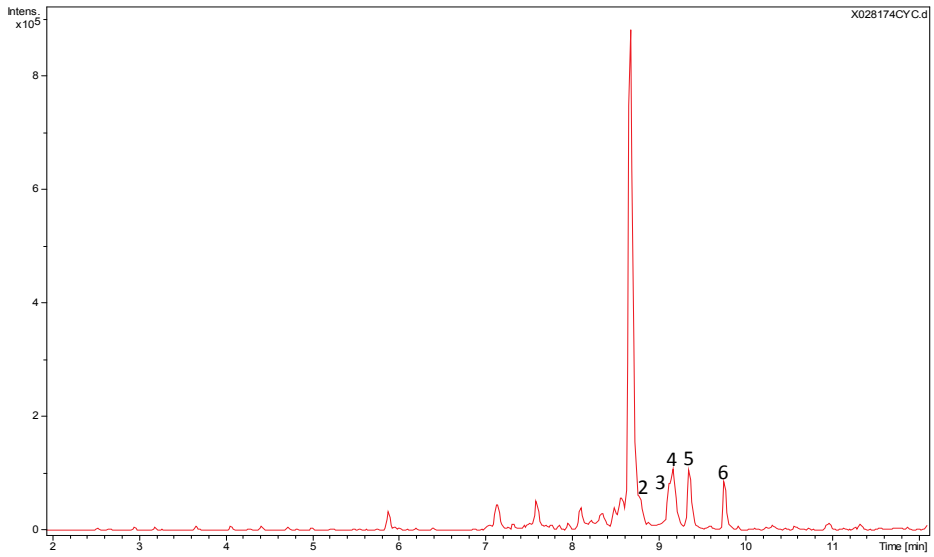
Linear crypto-thioester precursor of Cycloviolacin O2 (*cO2*, **1a**):

Sequence: H-CSCKSKVCYRNGIPCGESCVWIPCISSAIG-(Hnb)C(S*t*Bu)-G-NH_2

ESI-HRMS (*m/z*): $[MH]^+$ calcd. for $C_{149}H_{238}N_{41}O_{44}S_8$: 3561.5412, found: 3561.5415.
HPLC analysis: t_R = 3.64 min (Chromolith, gradient: 20-70% B/A over 5 min).
HPLC purification: Nucleosil, gradient: 40-50% B/A over 10 min, 70 °C.
Yield: 18%.



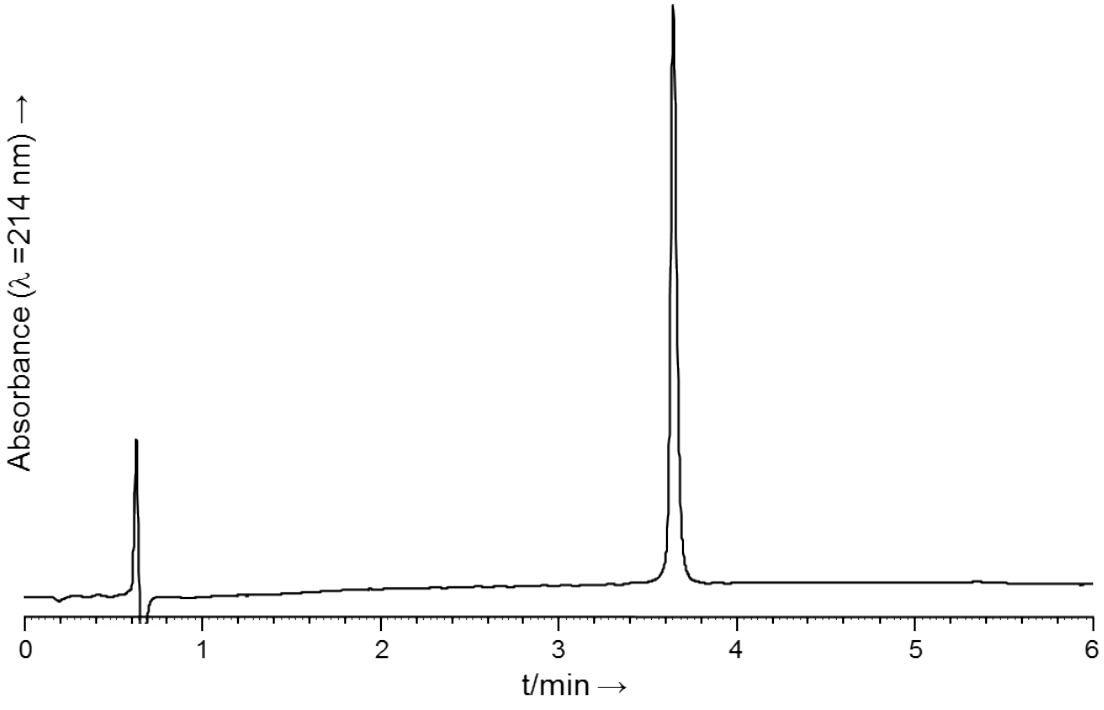
Supplementary figure S1: HPLC trace of crude **1a**.



Supplementary figure S2: LC/MS analysis of crude **1a** (base peak ion chromatogram).

Peak number (t_R (min))	$[MH]^+$ (*m/z*) calcd.	$[MH]^+$ (*m/z*) found	Attributed to
1 (8.68)	3561.5412	3561.5402	**1a**
2 (8.74)	3617.6038	3617.6002	**1a** + *t*Bu
3 (9.06)	3310.5014	3310.4997	Ac-[4-30]*cO2*-(Hnb)C(S*t*Bu)-G-NH_2
4 (9.22)	-	3616.5968	Not attributed
5 (9.36)	3617.6038	3617.6017	**1a** + *t*Bu
6 (9.77)	987.4280	987.4270	Ac-[25-30]*cO2*-(Hnb)C(S*t*Bu)-G-NH_2

Supplementary table S3: Attribution of the main peaks observed during LC/MS analysis of crude **1a**.

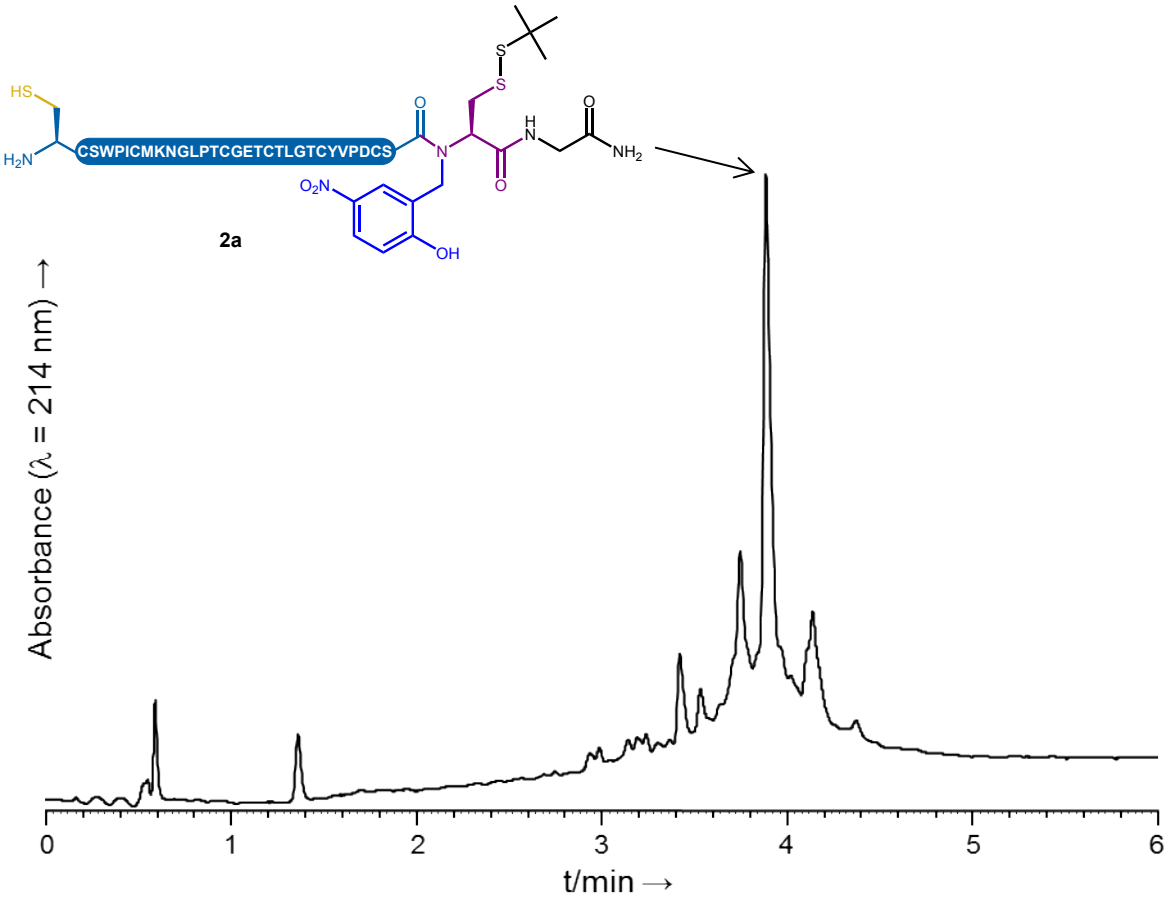


Supplementary figure S3: HPLC trace of purified **1a**.

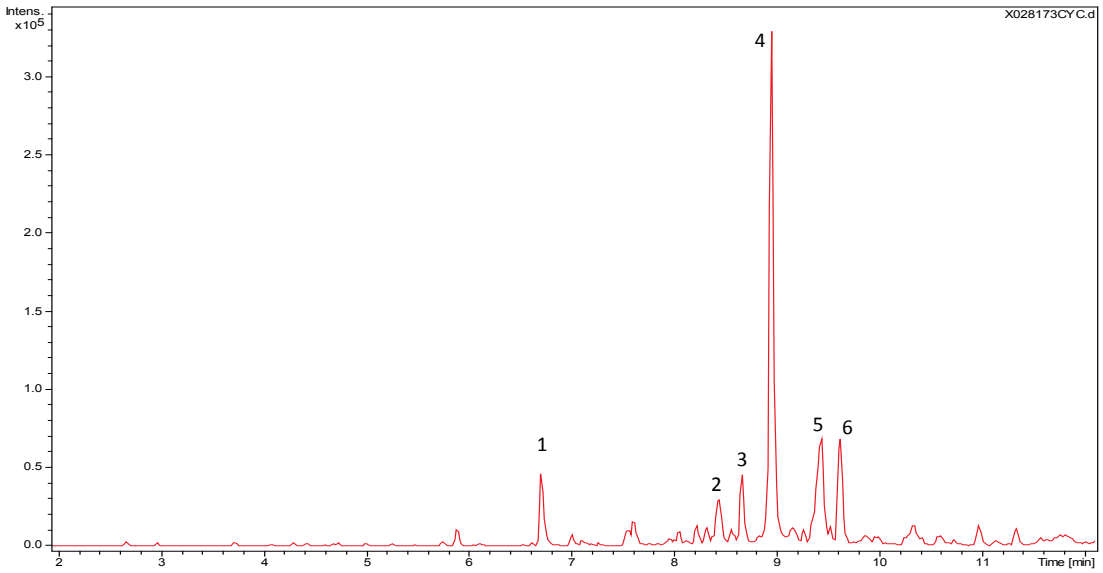
Linear crypto-thioester precursor of Cter M (**2a**):

Sequence: H-CSWPICMKNGLPTCGETCTLGTCYVPDCS-(Hnb)C(S*t*Bu)-G-NH_2

ESI-HRMS (*m/z*): $[MH]^+$ calcd. for $C_{144}H_{223}N_{36}O_{46}S_9$: 3480.3704, found: 3480.3686.
HPLC analysis: t_R = 3.88 min (Chromolith, gradient: 20-60% B/A over 5 min).
HPLC purification: Nucleosil, gradient: 40-50% B/A over 10 min.
Yield: 10%.



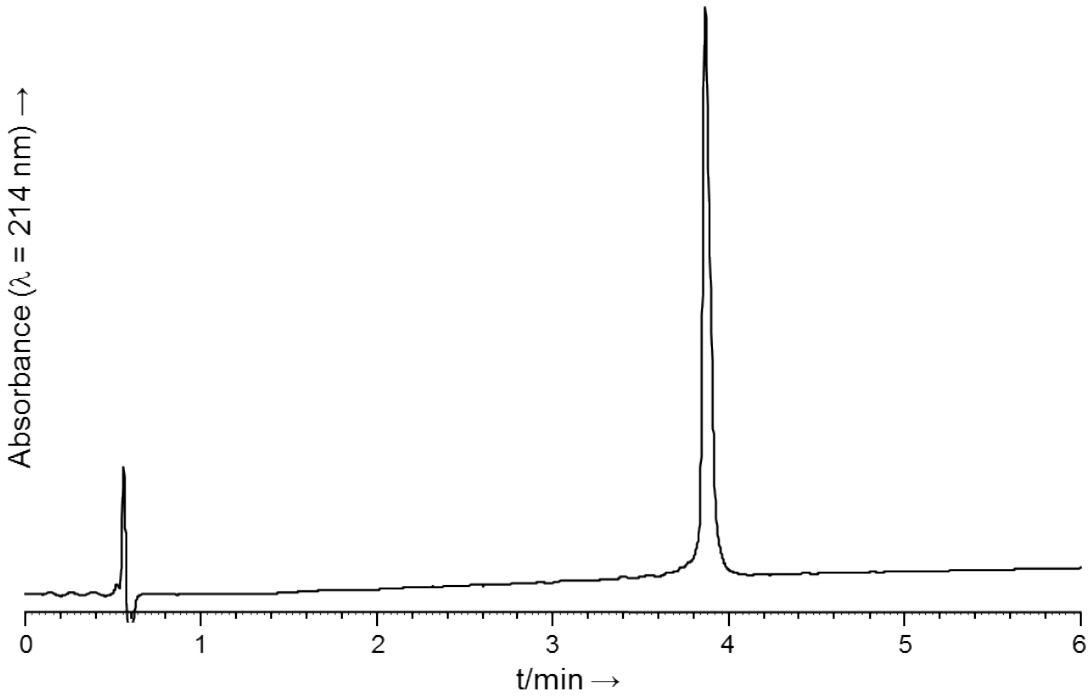
Supplementary figure S4: HPLC trace of crude **2a**.



Supplementary figure S5: LC/MS analysis of crude **2a** (base peak ion chromatogram).

Peak number (t_R (min))	$[MH]^+$ (*m/z*) calcd.	$[MH]^+$ (*m/z*) found	Attributed to
1 (6.72)	607.1678	607.1656	H-CS-(Hnb)C(S*t*Bu)-G-NH_2
2 (8.44)	3480.3704	3480.3675	Same mass as **2a**
3 (8.66)	3496.3653	3496.3624	**2a** with oxidized methionine
4 (8.95)	3480.3704	3480.3674	**2a**
5 (9.43)	3536.4330	3536.4304	**2a** + *t*Bu
6 (9.62)	2091.7301	2091.7275	Ac-[14-29]*CterM*-(Hnb)C(S*t*Bu)-G-NH_2

Supplementary table S4: Attribution of the main peaks observed during LC/MS analysis of crude **2a**.

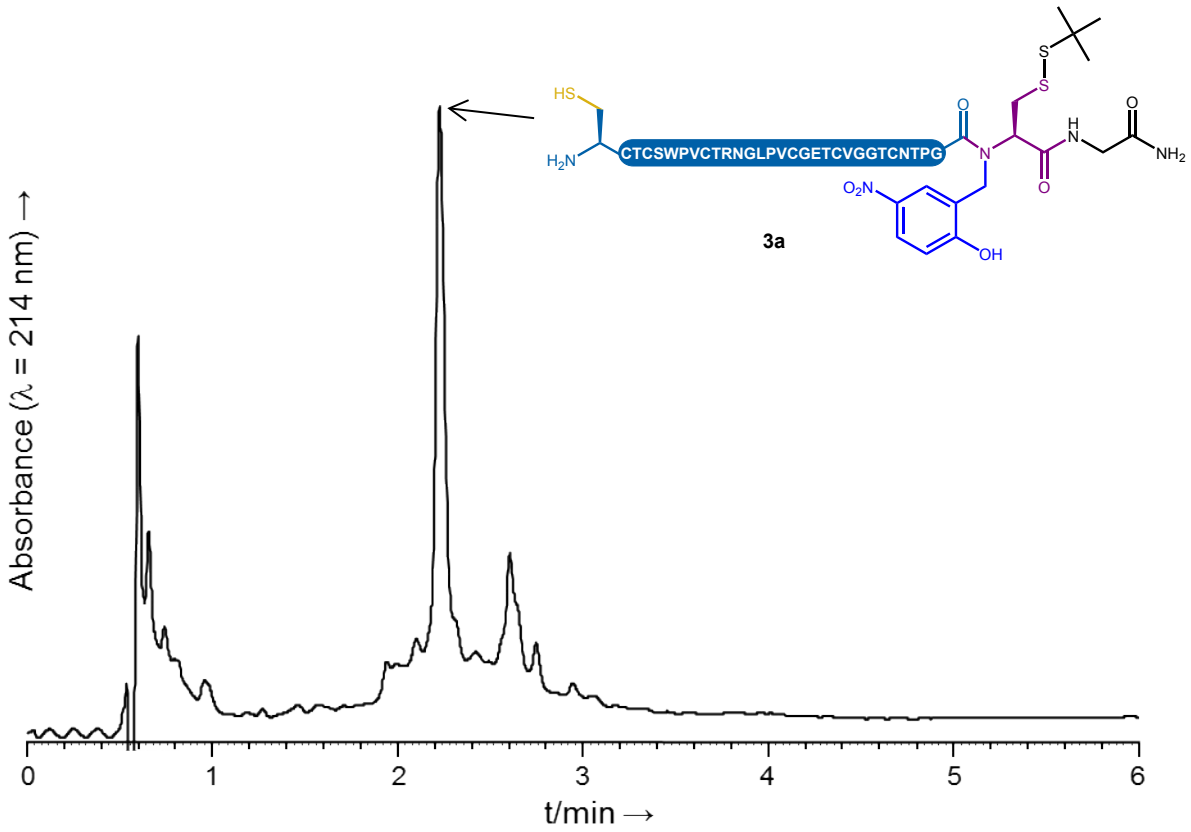


Supplementary figure S6: HPLC trace of purified **2a**.

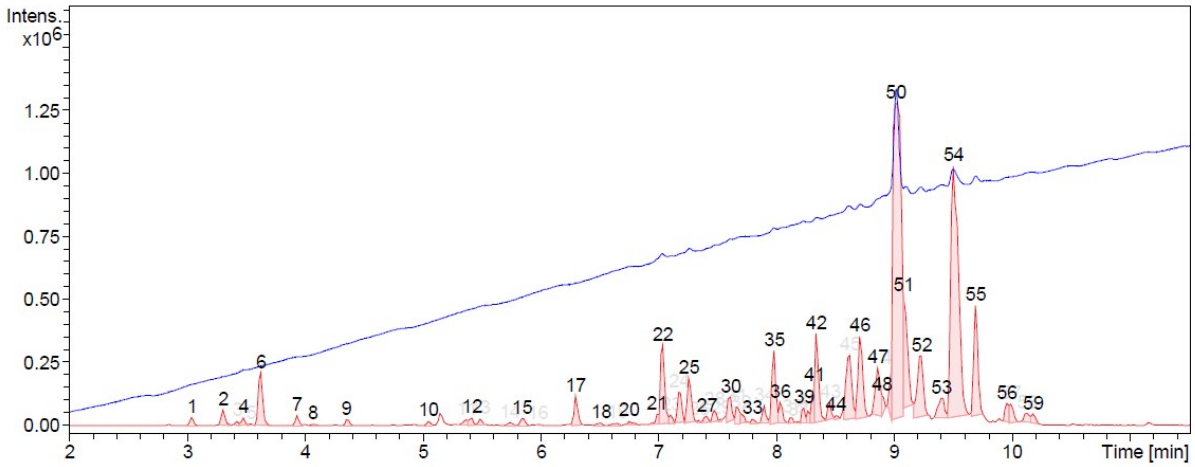
Linear crypto-thioester precursor of Kalata B1 (**3a**):

Sequence: H-CTCSWPVCTRNGLPVCGETCVGGTCNTPG-(Hnb)C(S*t*Bu)-G-NH_2

ESI-HRMS (*m/z*): $[MH]^+$ calcd. for $C_{133}H_{210}N_{39}O_{44}S_8$: 3313.3160, found: 3313.3153.
HPLC analysis: t_R = 2.10 min (Chromolith, gradient: 30-60% B/A over 5 min).
HPLC purification: Nucleosil, gradient: 35-45% B/A over 10 min.
Yield: 16%.



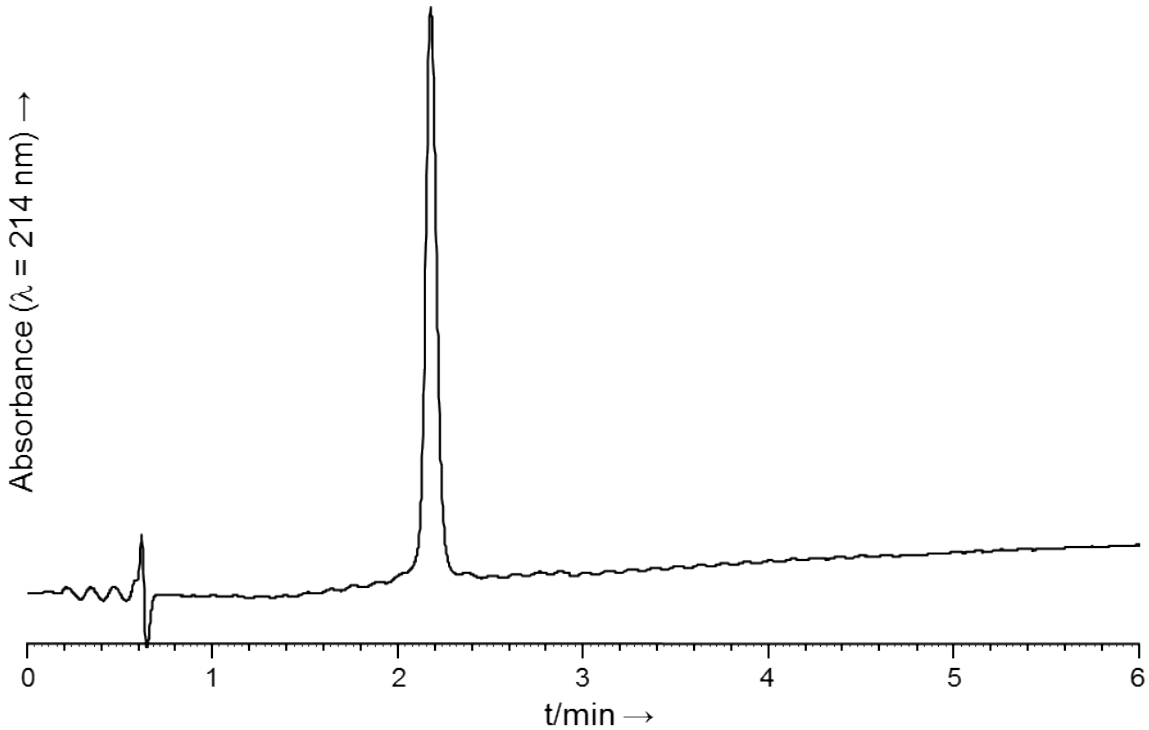
Supplementary figure S7: HPLC trace of crude **3a**.



Supplementary figure S8: LC/MS analysis of crude **3a**. Blue trace: UV (λ = 214 nm); red trace: base peak ion chromatogram.

Peak number (t_R (min))	$[MH]^+$ (*m/z*) calcd.	$[MH]^+$ (*m/z*) found	Attributed to
50 (9.01)	3313.3160	3313.3153	**3a**
54[a] (9.50)	3369.3786	3369.3777	**3a** + *t*Bu
55 (9.68)	3369.3786	3369.3764	**3a** + *t*Bu

Supplementary table S5: Attribution of the main peaks observed during LC/MS analysis of crude **3a**. [a]: Note that the large LC/MS peak is not representative of the actual proportion of **3a** / **3a** + *t*Bu, see UV trace for quantification.

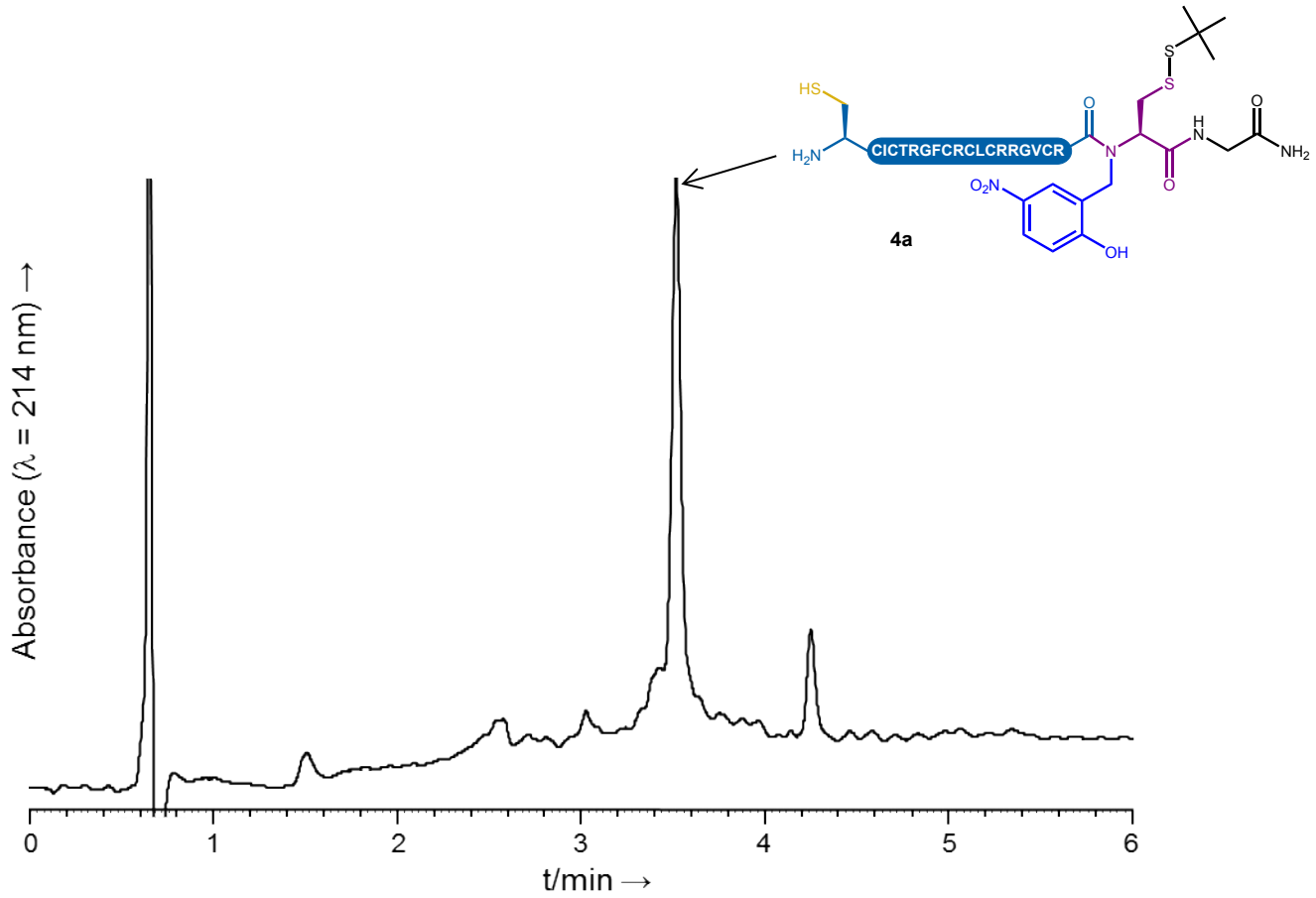


Supplementary figure S9: HPLC trace of purified **3a**.

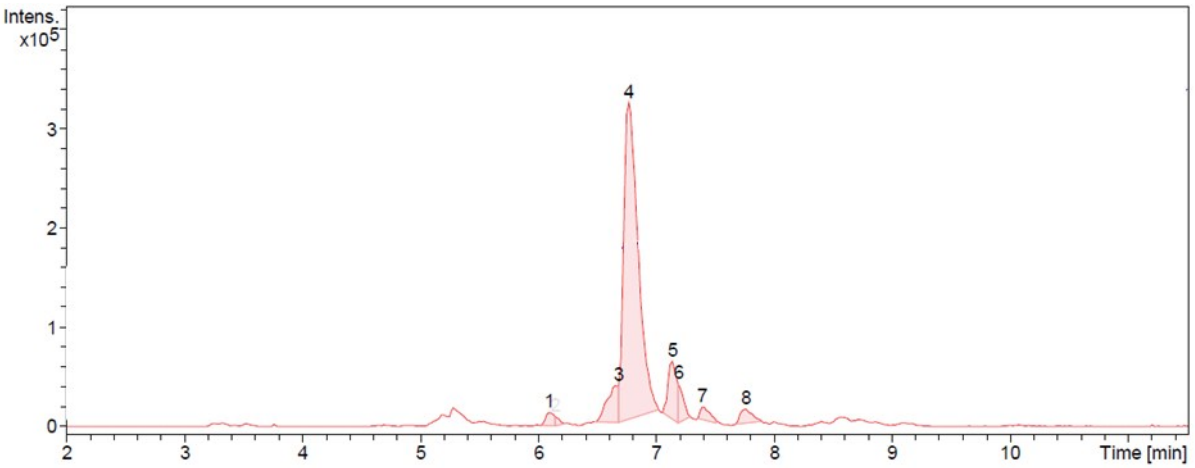
Linear crypto-thioester precursor of RTD-1 (**4a**):

Sequence: H-CICTRGFCRCLCRRGVCR-(Hnb)C(S*t*Bu)-G-NH_2

ESI-HRMS (*m/z*): $[MH]^+$ calcd. for $C_{98}H_{168}N_{37}O_{24}S_8$: 2503.0829, found: 2503.0873.
HPLC analysis: t_R = 3.52 min (Chromolith, gradient: 20-50% B/A over 5 min).
HPLC purification: Nucleosil, gradient: 30-35% B/A over 5 min.
Yield: 11%.



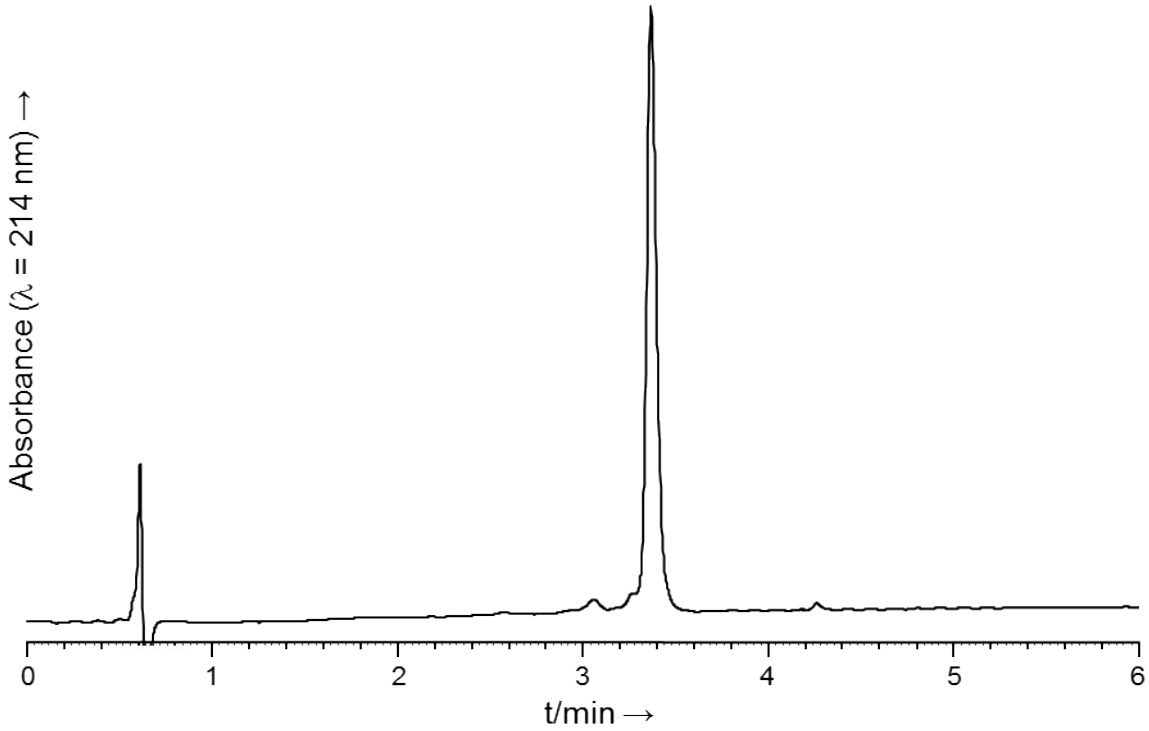
Supplementary figure S10: HPLC trace of crude **4a**.



Supplementary figure S11: LC/MS analysis of crude **4a** (base peak ion chromatogram).

Peak number (t_R (min))	$[MH]^+$ (*m/z*) calcd.	$[MH]^+$ (*m/z*) found	Attributed to
4 (6.76)	2503.0829	2503.0873	**4a**
5 (7.14)	615.2383	615.2369	Ac-R-(Hnb)C(S*t*Bu)-G-NH_2
7 (7.39)	1505.6421	1505.6435	Ac-[10-18]*RTD-1*-(Hnb)C(S*t*Bu)-G-NH_2
8 (7.76)	1968.8422	1968.8445	Ac-[6-18]*RTD-1*-(Hnb)C(S*t*Bu)-G-NH_2

Supplementary table S6: Attribution of the main peaks observed during LC/MS analysis of crude **4a**.

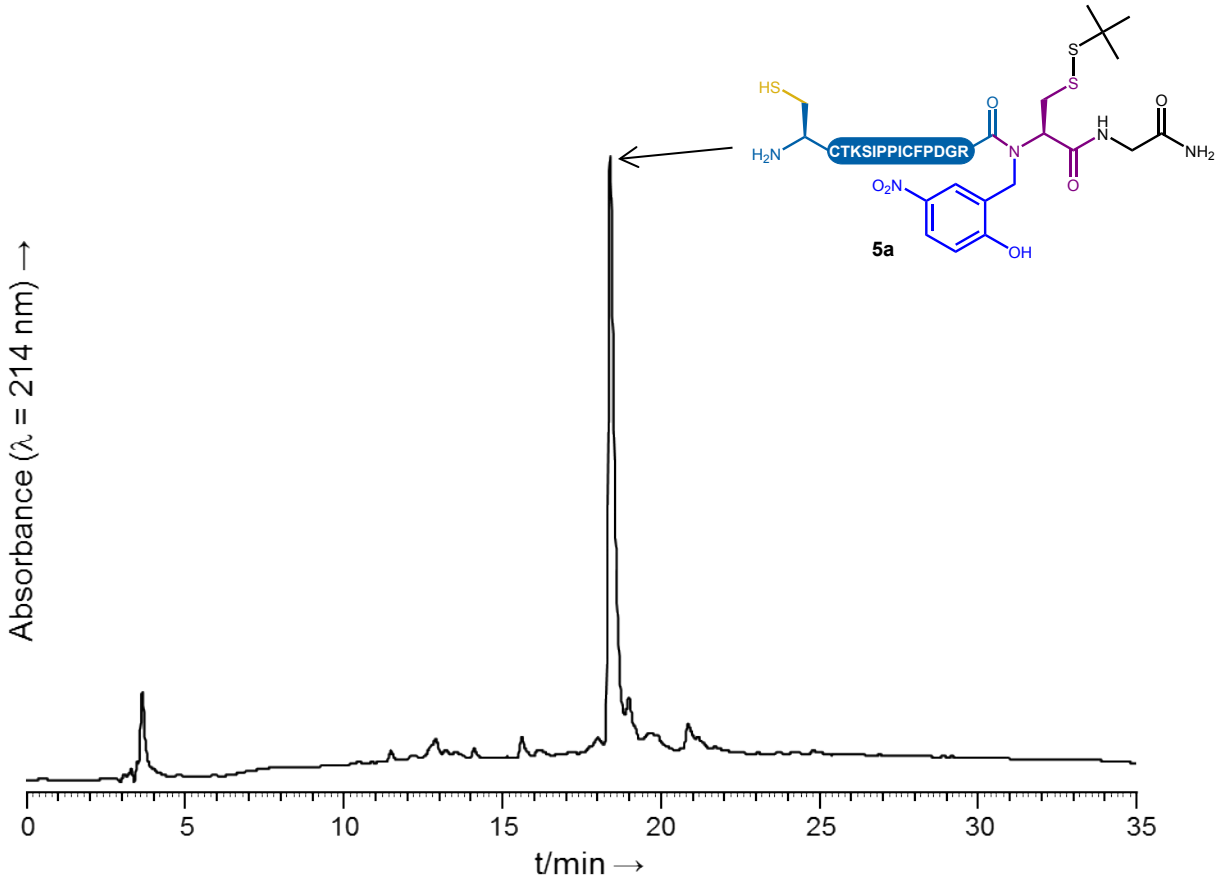


Supplementary figure S12: HPLC trace of purified **4a**.

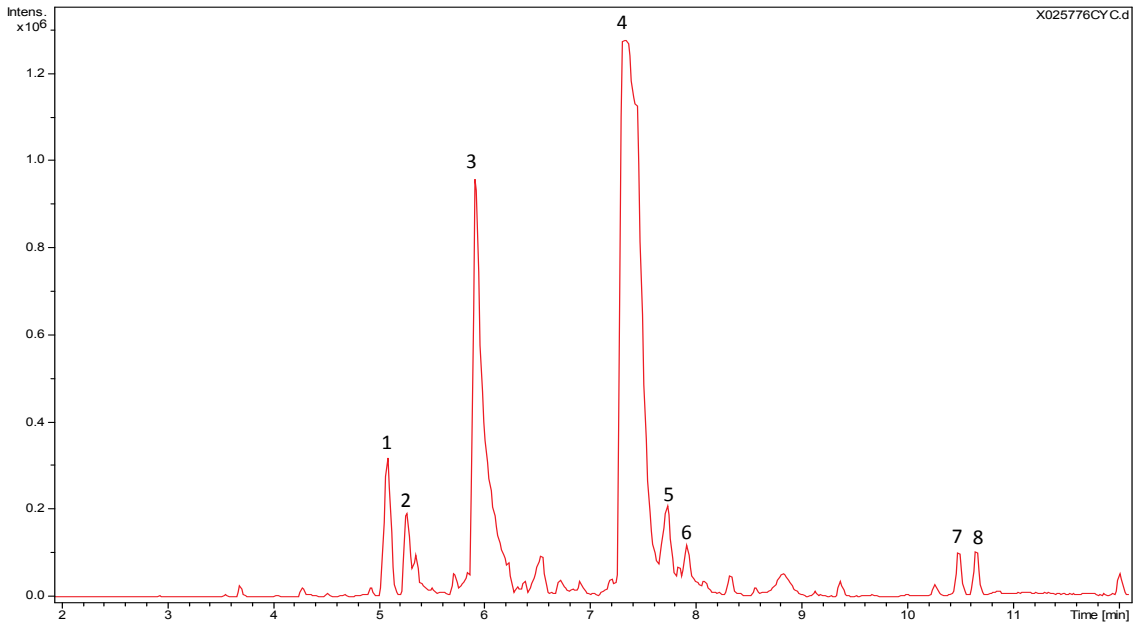
Linear crypto-thioester precursor of SFTI-1 (**5a**):

Sequence: H-CTKSIPPICFPDGR-(Hnb)C(S*t*Bu)-G-NH_2

ESI-HRMS (*m/z*): $[MH]^+$ calcd. for $C_{83}H_{131}N_{22}O_{23}S_4$: 1931.8640, found: 1931.8651.
HPLC analysis: t_R = 18.45 min (Nucleosil, gradient: 20-60% B/A over 30 min, 70 °C). HPLC analysis was performed at high temperature due to the presence of large peaks when analysing **5a** on a chromolith column at room temperature.
HPLC purification: Nucleosil, gradient: 35-40% B/A over 5 min.
Yield: 21%.



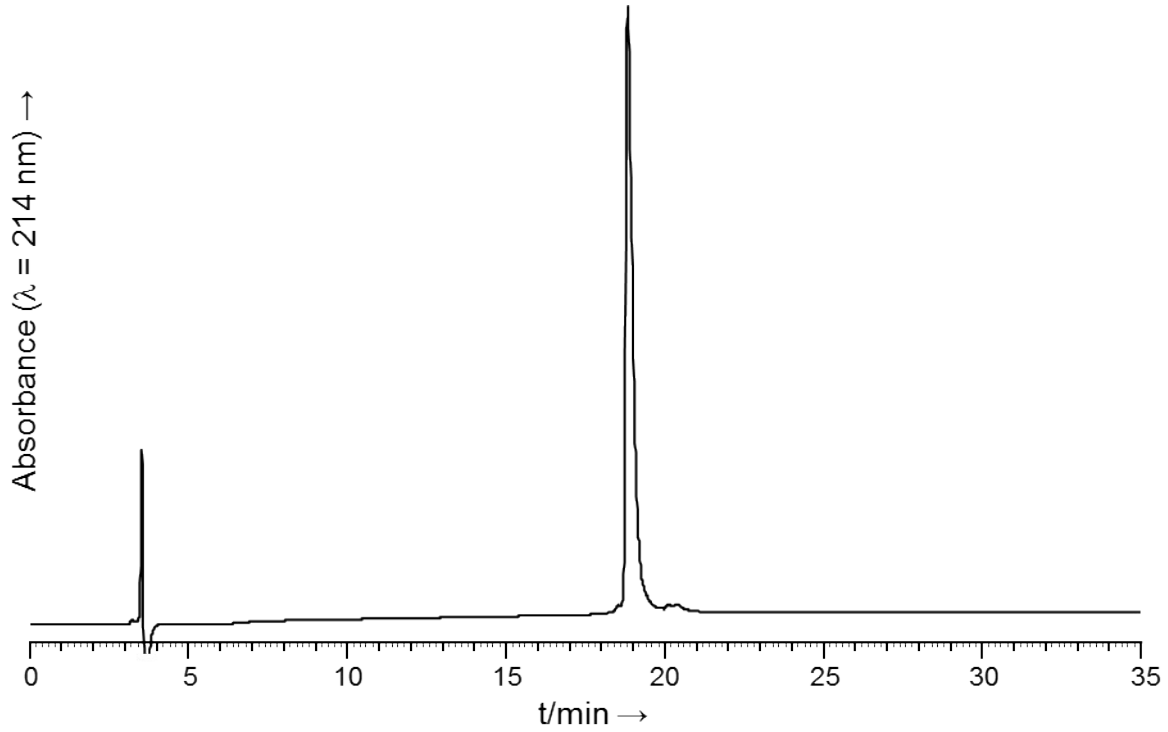
Supplementary figure S13: HPLC trace of crude **5a**.



Supplementary figure S14: LC/MS analysis of crude **5a** (base peak chromatogram).

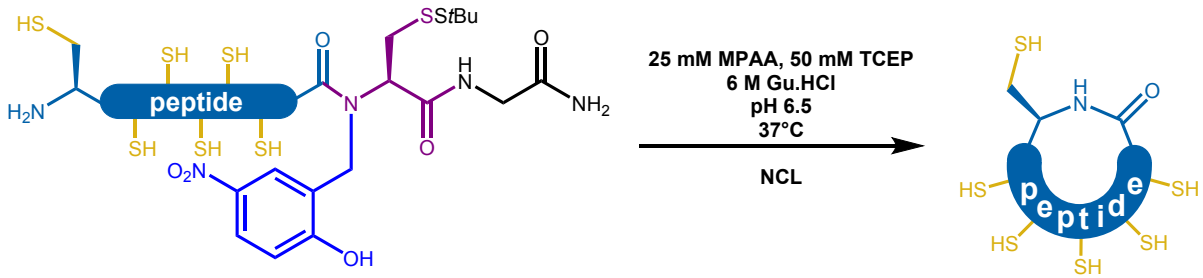
Peak number (t_R (min))	$[MH]^+$ (*m/z*) calcd.	$[MH]^+$ (*m/z*) found	Attributed to
1 (5.09)	-	574.1854	Not attributed
2 (5.28)	-	1533.7551	Not attributed
3[a] (5.93)	-	1320.6322	Not attributed
4 (7.34-7.41)	1931.8640	1931.8631	**5a**
5 (7.73)	1929.8484	1929.8476	**5a** + 1 disulfide bond
6 (7.92)	1987.9266	1987.9245	**5a** + *t*Bu
7 (10.49)	-	385.0586	Not attributed
8 (10.66)	-	323.0236	Not attributed

Supplementary table S7: Attribution of the main peaks observed during LC/MS analysis of crude **5a**. [a]: Note that the large LC/MS peak is not representative of the actual quantity.



Supplementary figure S15: HPLC trace of purified **5a**.

4. Peptide cyclization *via* intramolecular NCL



1a-5a **1b-5b**

Supplementary scheme S4: Syntheses of peptides **1b-5b** *via* intramolecular NCL.

General procedure:

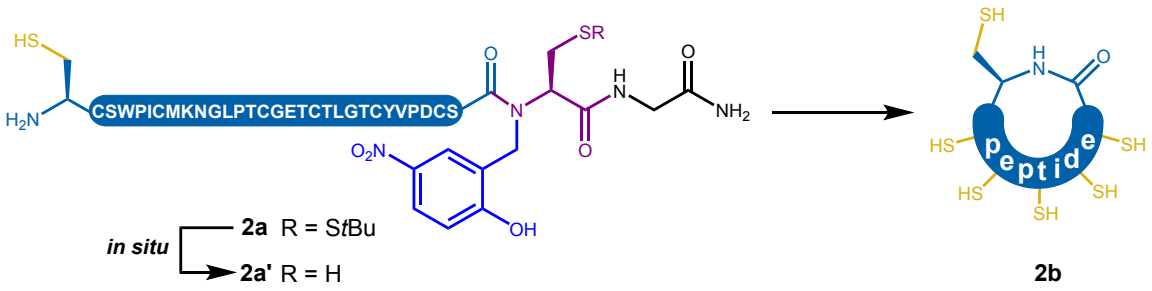
A deoxygenated[a] 0.2 M pH 6.5 sodium phosphate buffer containing 6 M guanidine hydrochloride, 100 mM MPAA and 50 mM TCEP was added to HPLC-purified dry peptides **1a-5a** (final concentration 1 mM) under argon. The ligations were carried out at 37°C and monitored by RP-HPLC. After completion, the reaction mixtures were acidified to pH 1 using 3% TFA in water. These solutions were then extracted with diethyl ether (× 4) to remove MPAA. In case of disulfide formation due to contamination with oxygen, TCEP was added (final concentration 100 mM) and pH was adjusted to 5.0 using a 10 M NaOH solution; after 20 min, pH was adjusted to 1 with 3% TFA in water. The ligation products were purified by semi-preparative RP-HPLC. All the HPLC purification runs started with a 15 min plateau under the gradient initial conditions; this point is crucial to ensure the complete removal of salts (Gu.HCl) and to secure the NCL yield calculations by weigh, which is required for peptides **4b** and **5b** not containing Tyr or Trp residues.

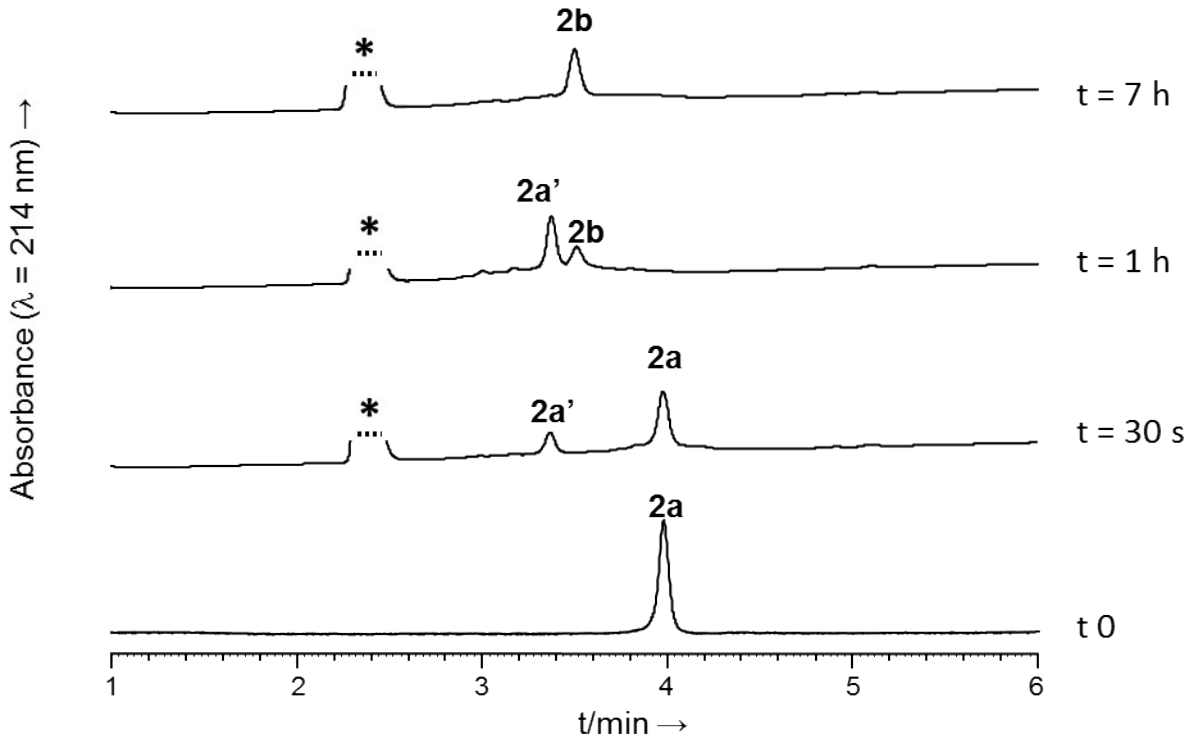
For **3a** and **5a**, the intramolecular cyclization was also conducted at lower peptide concentration (0.5 mM for **3a** and 0.1 mM for **5a**), in order to minimize the formation of cyclic dimers **3c** and **5c**.

[a]: deoxygenation was performed through four consecutive vacuum/argon cycles.

Examples of reaction monitoring for the cyclization of **2b** and **4b**:

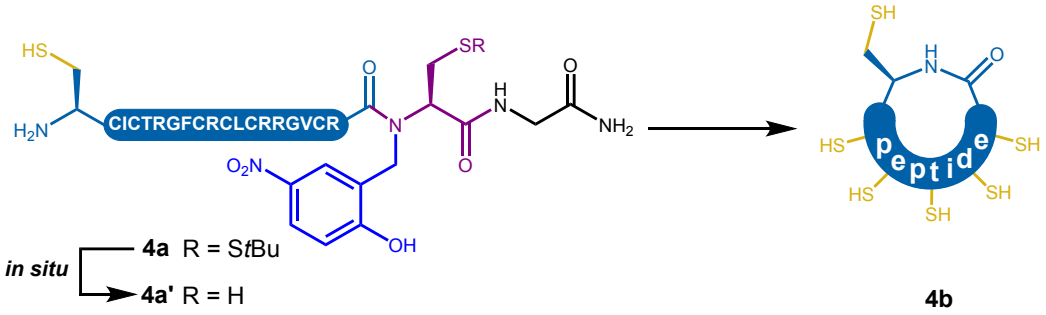
Reduced form of Cter M (2b):

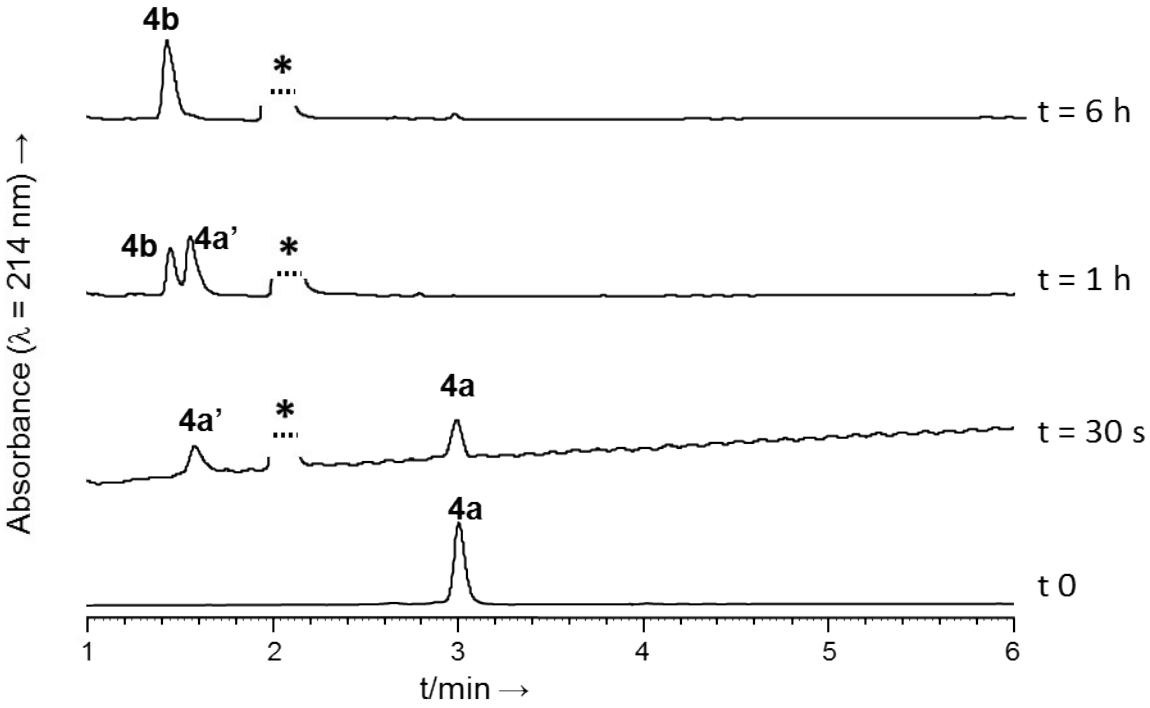




Supplementary figure S16: Analytical HPLC monitoring of the NCL-based cyclization of **2a**. Chromolith column, gradient: 20-60% B/A over 5 min. (* = MPAA).

Reduced form of RTD-1 (4b):



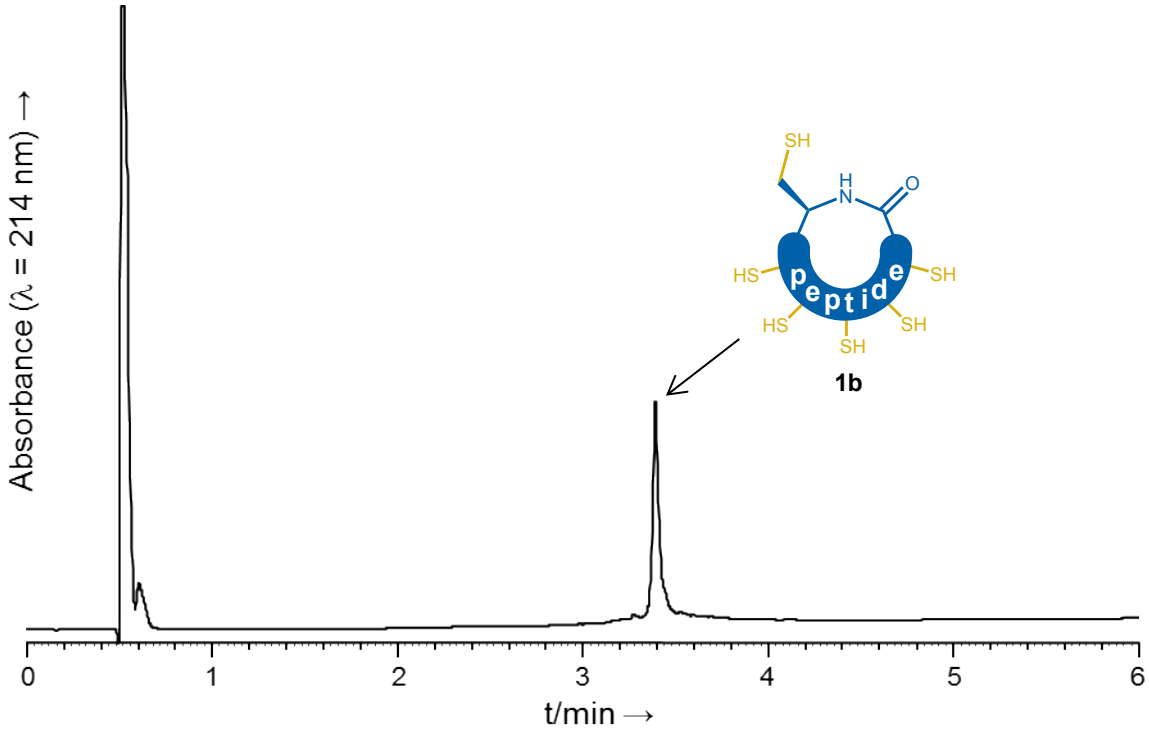


Supplementary figure S17: Analytical HPLC monitoring of the NCL-based cyclization of **4a**. Chromolith column, gradient: 25-45% B/A over 5 min. (* = MPAA).

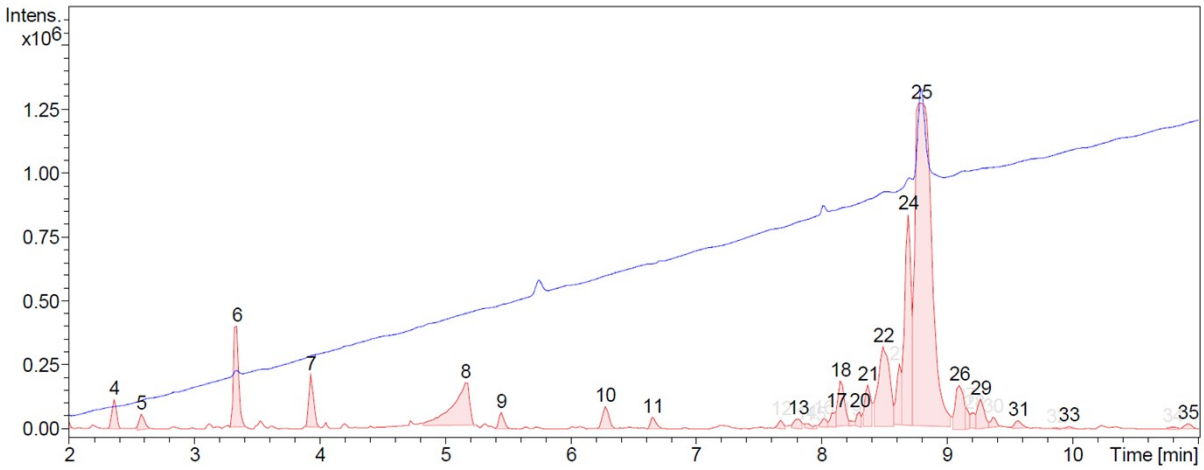
Reduced form of Cycloviolacin O2 (**1b**):

Sequence: cyclo(CSCKSKVCYRNGIPCGESCVWIPCISSAIG)

ESI-HRMS (*m/z*): $[MH]^+$ calcd. for $C_{133}H_{214}N_{37}O_{39}S_6$: 3145.4224, found: 3145.4175.
HPLC analysis: t_R = 3.41 min (Chromolith, gradient: 20-70% B/A over 5 min).
HPLC purification: Nucleosil, gradient: 30% B/A over 15 min then 30-40% B/A over 10 min.
Yield: 65%.



Supplementary figure S18: HPLC trace of **1b** after work-up.

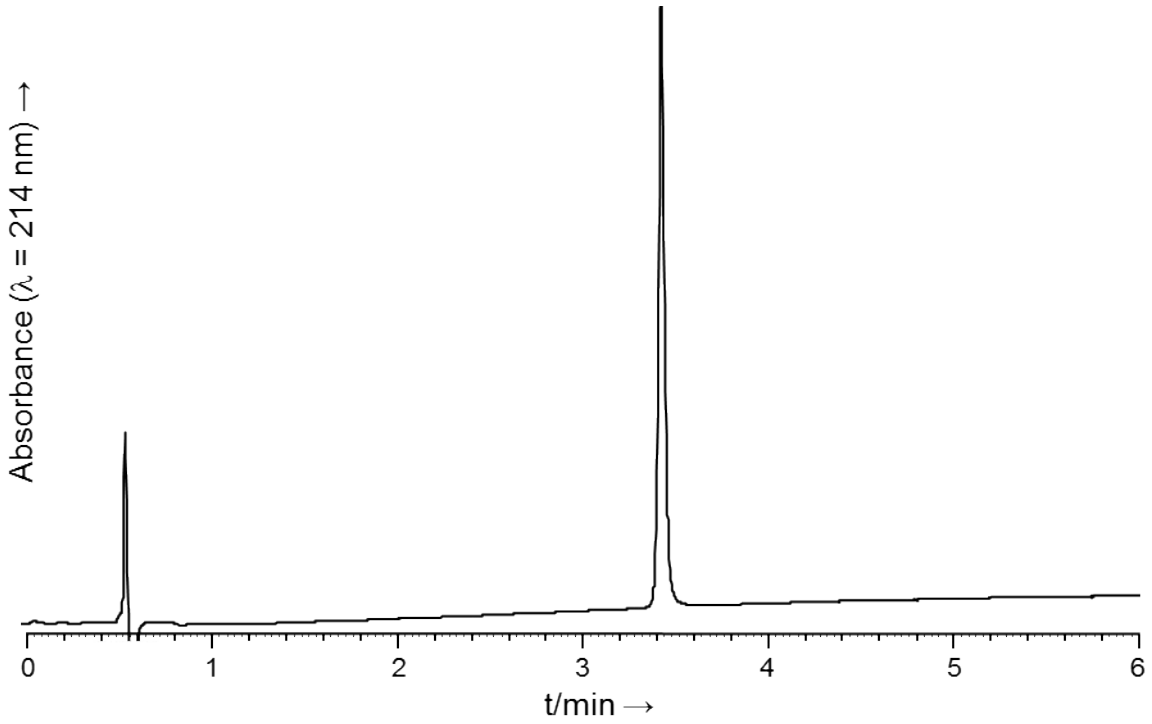


Supplementary figure S19: LC/MS analysis of crude **1b** after work-up. Blue trace: UV (λ = 214 nm); red trace: base peak ion chromatogram.

Peak number (t_R (min))	$[MH]^+$ (*m/z*) calcd.	$[MH]^+$ (*m/z*) found	Attributed to
24[a] (8.69)	-	3149.4145	Not attributed
25 (8.79)	3145.4224	3145.4175	**1b**
27 (9.14)	6289.8370	6289.8281	Cyclic dimer **S6**[b]

Supplementary table S8: Attribution of the main peaks observed during LC/MS analysis of crude **1b** after work-up. [a]: Note that the large LC/MS peak is not representative of the actual quantity.

[b]: **S6** = cyclo(CSCKSKVCYRNGIPCGESCVWIPCISSAIGCSCKSKVCYRNGIPCGESCVWIPCISSAIG).

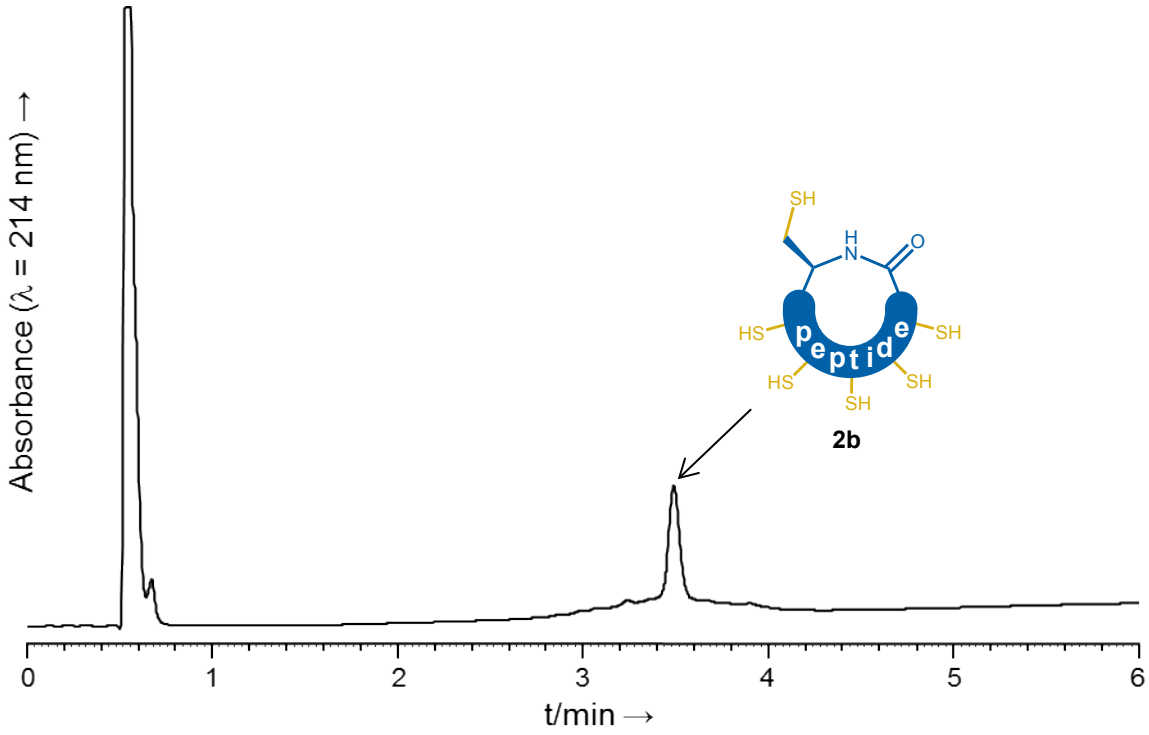


Supplementary figure S20: HPLC trace of pure **1b**.

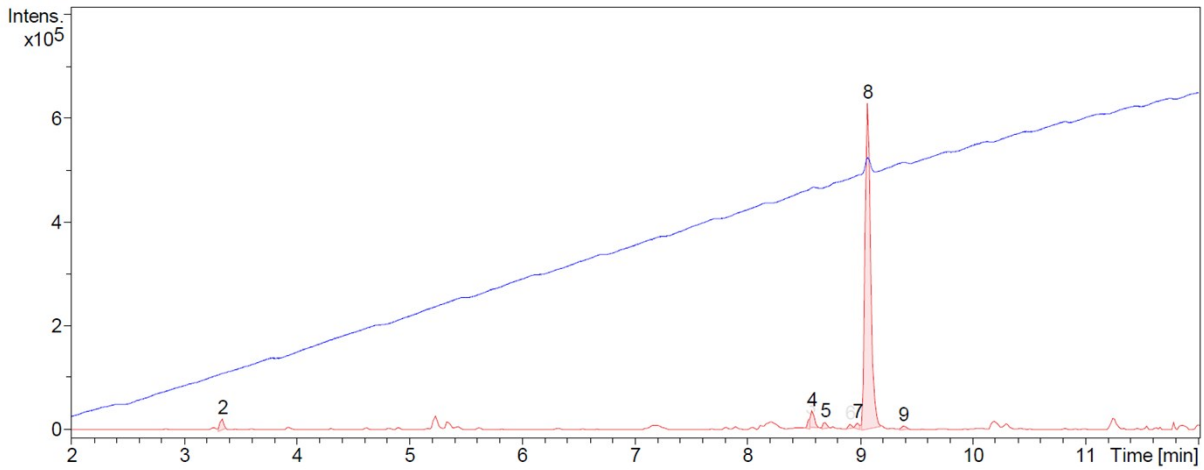
Reduced form of Cter M (**2b**):

Sequence: cyclo(CSWPICMKNGLPTCGETCTLGTCYVPDCS).

ESI-HRMS (*m/z*): $[MH]^+$ calcd. for $C_{128}H_{199}N_{32}O_{41}S_7$: 3064.2516, found: 3064.2467.
HPLC analysis: t_R = 3.49 min (Chromolith, gradient: 20-60% B/A over 5 min).
HPLC purification: Nucleosil, gradient: 30% B/A over 15 min then 30-40% B/A over 10 min.
Yield: 59%.



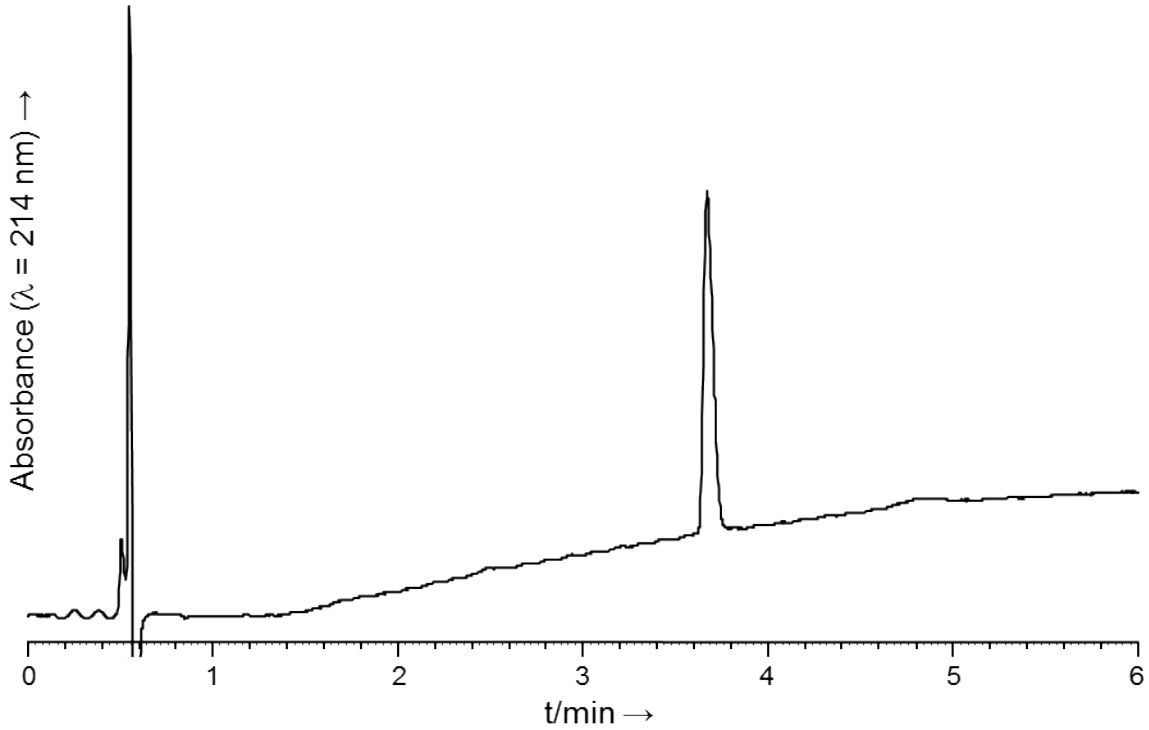
Supplementary figure S21: HPLC trace of crude **2b** after work-up.



Supplementary figure S22: LC/MS analysis of crude **2b** after work-up. Blue trace: UV (λ = 214 nm); red trace: base peak ion chromatogram.

Peak number (t_R (min))	$[MH]^+$ (*m/z*) calcd.	$[MH]^+$ (*m/z*) found	Attributed to
4 (8.57)	3080.2465	3080.2385	**2b** with oxidized methionine
8 (9.06)	3064.2516	3064.2467	**2b**

Supplementary table S9: Attribution of the main peaks observed during LC/MS analysis of crude **2b** after work-up.

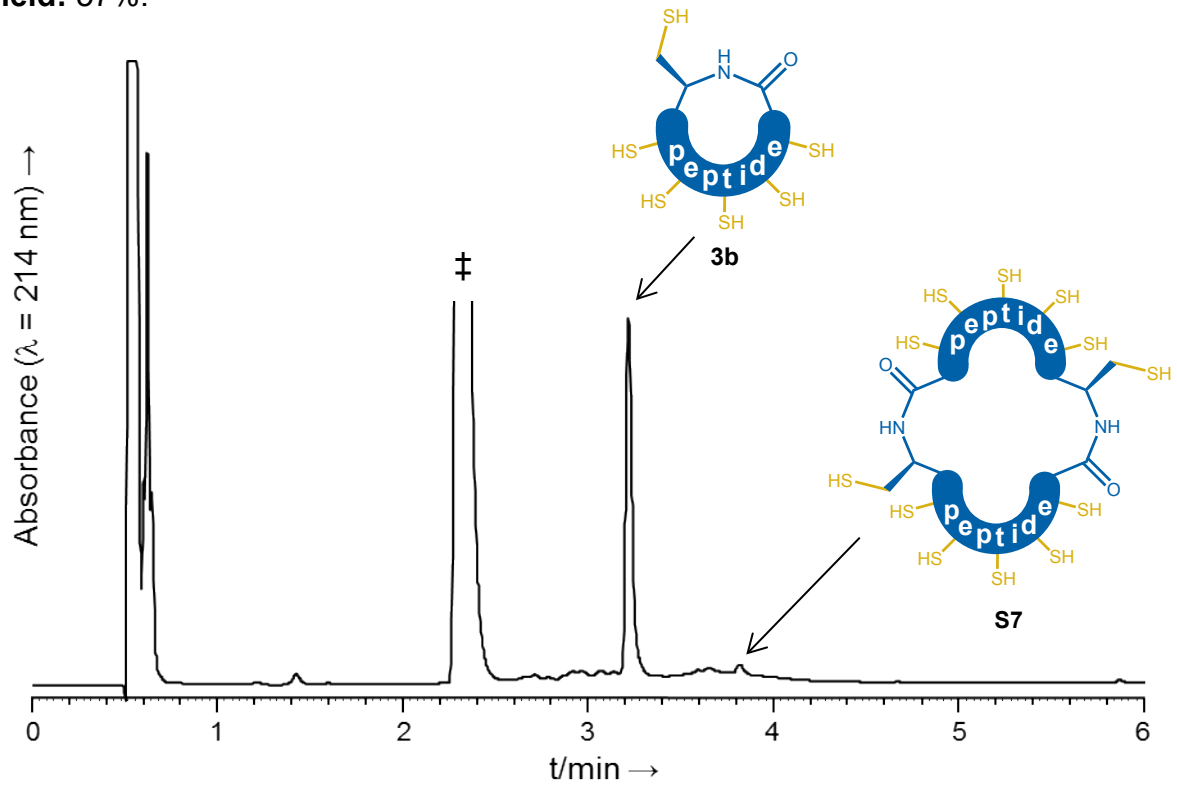


Supplementary figure S23: HPLC trace of purified **2b**.

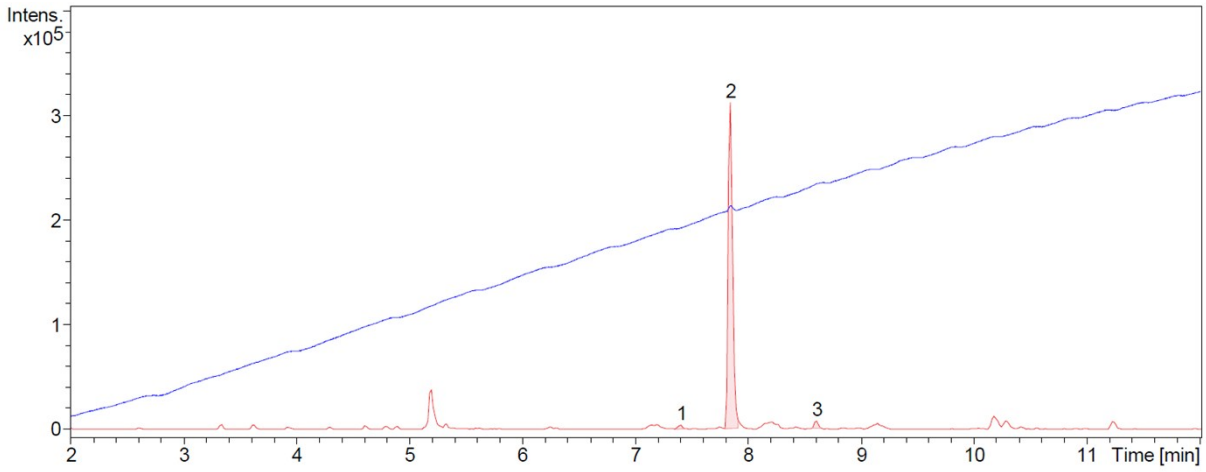
Reduced form of Kalata B1 (**3b**):

Sequence: cyclo(CTCSWPVCTRNGLPVCGETCVGGTCNTPG).

ESI-HRMS (*m/z*): $[MH]^+$ calcd. for $C_{117}H_{186}N_{35}O_{39}S_6$: 2897.1976, found: 2897.1973.
HPLC analysis: t_R = 3.24 min (Chromolith, gradient: 20-50% B/A over 5 min).
HPLC purification: Nucleosil, gradient: 25% B/A over 15 min then 25-35% B/A over 10 min.
Yield: 57%.



Supplementary figure S24: HPLC trace of crude **3b** (NCL at 1 mM) after work-up (‡: MPAA).

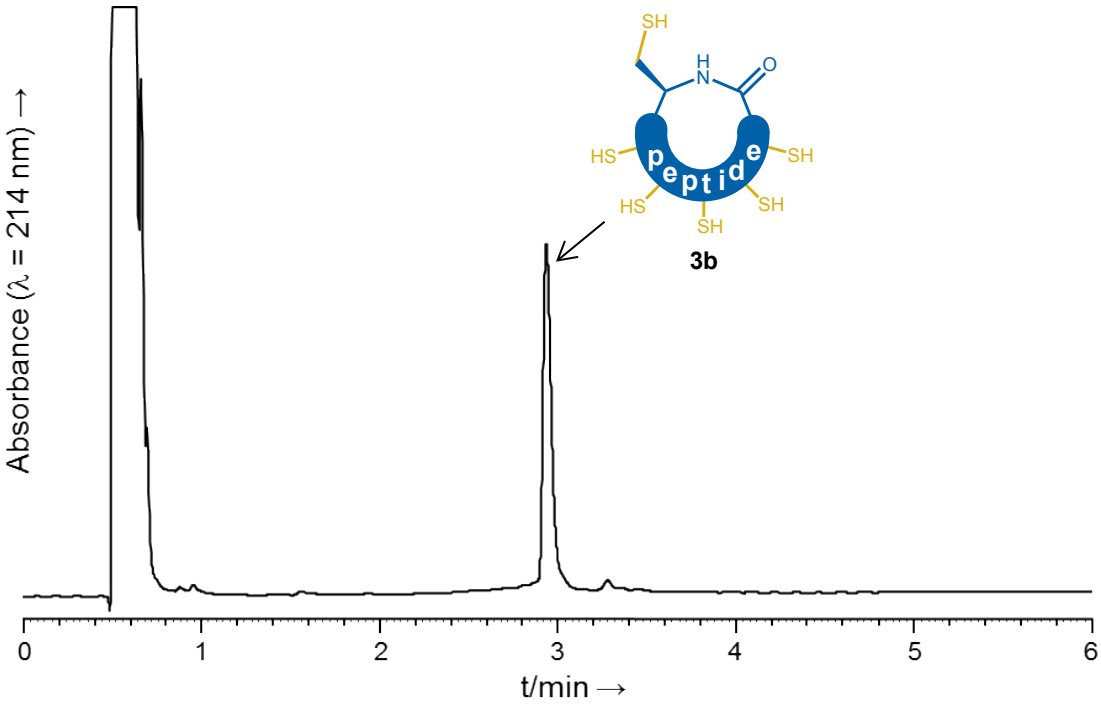


Supplementary figure S25: LC/MS analysis of crude **3b** (NCL at 1 mM) after work-up. Blue trace: UV (λ = 214 nm); red trace: base peak ion chromatogram.

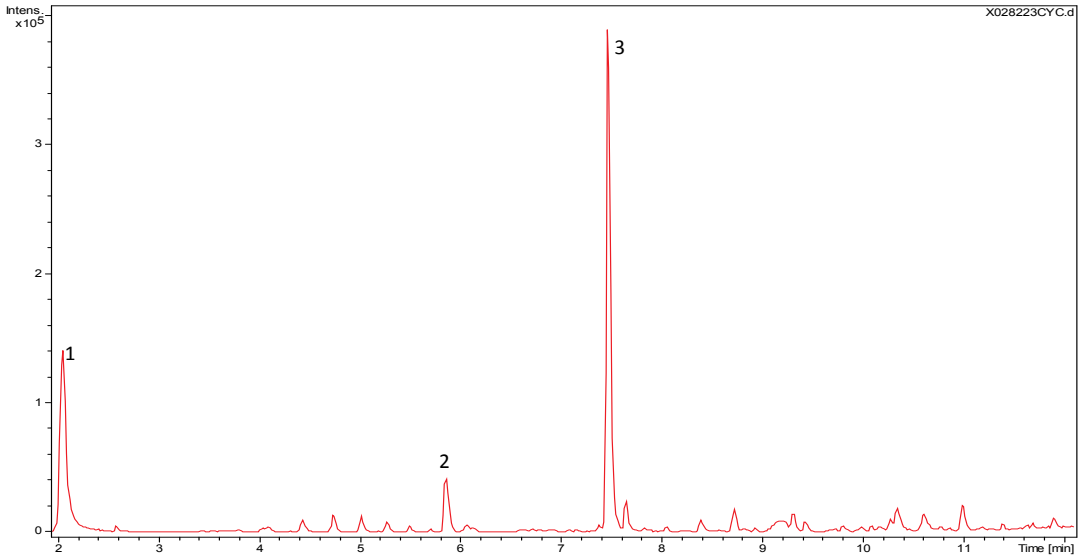
Peak number (t_R (min))	$[MH]^+$ (*m/z*) calcd.	$[MH]^+$ (*m/z*) found	Attributed to
2 (7.85)	2897.1976	2897.1973	**3b**
3 (8.60)	5793.3874	5793.3902	Cyclic dimer **S7**[a]

Supplementary table S10: Attribution of the main peaks observed during LC/MS analysis of crude **3b** (NCL at 1 mM) after work-up.

[a]: **S7** = cyclo(CTCSWPVCTRNGLPVCGETCVGGTCNTPGCTCSWPVCTRNGLPVCGETCVGGTCNTPG).



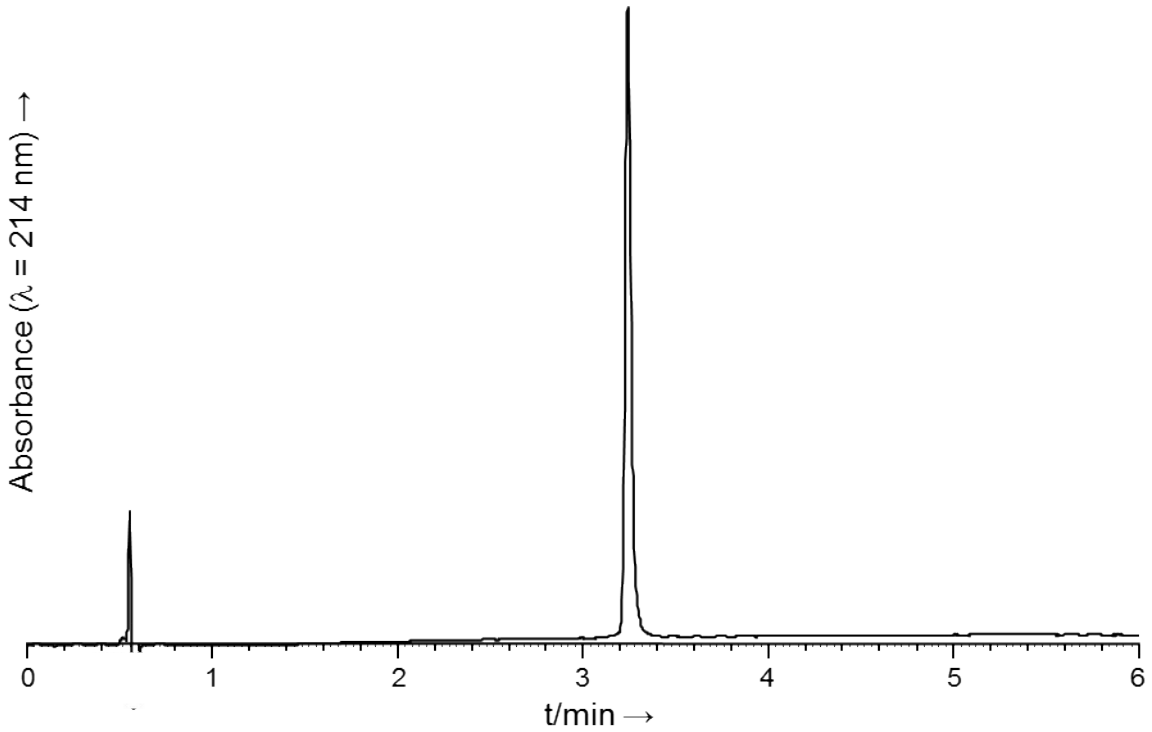
Supplementary figure S26: HPLC trace of crude **3b** (NCL at 0.5 mM) after work-up.



Supplementary figure S27: LC/MS analysis of crude **3b** (NCL at 0.5 mM) after work-up.Red trace: base peak ion chromatogram.

Peak number (t_R (min))	$[MH]^+$ (*m/z*) calcd.	$[MH]^+$ (*m/z*) found	Attributed to
1 (2.05)	-	265.0288	Not attributed
2 (5.86)	-	289.0341	Not attributed
3 (7.48)	2897.1976	2897.1949	**3b**

Supplementary table S11: Attribution of the main peak observed in LC/MS analysis of crude **3b** (NCL at 0.5 mM) after work-up.

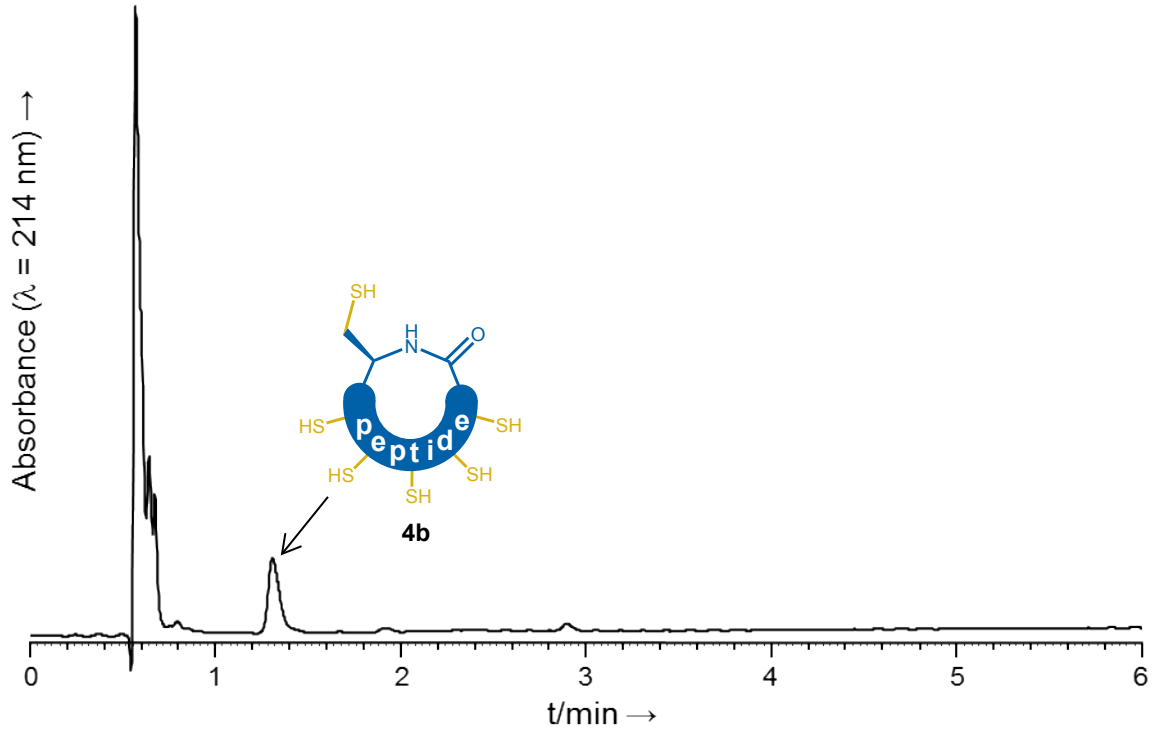


Supplementary figure S28: HPLC trace of purified **3b**.

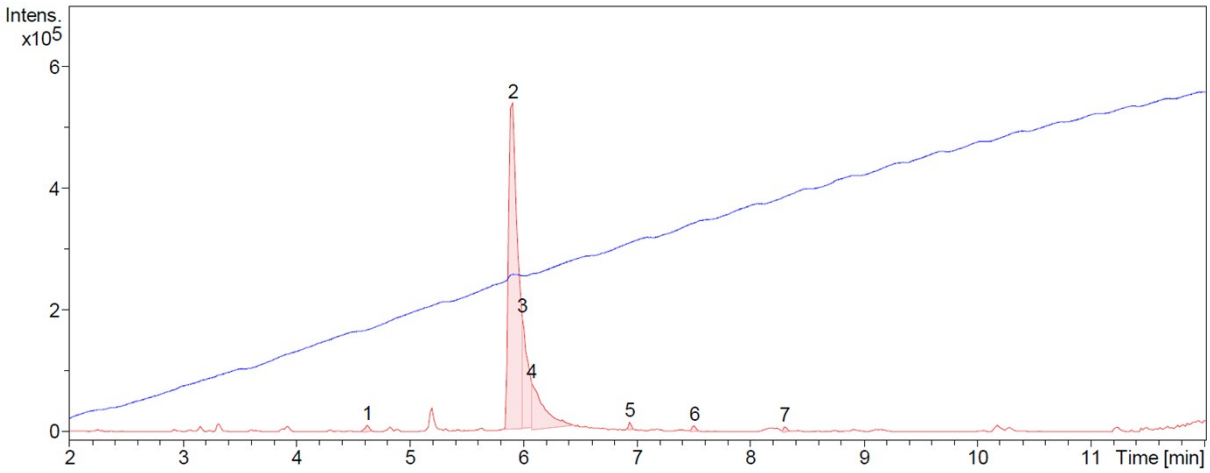
Reduced form of RTD-1 (**4b**):

Sequence: cyclo(CICTRGFCRCLCRRGVCR).

ESI-HRMS (*m/z*): $[MH]^+$ calcd. for $C_{82}H_{144}N_{33}O_{19}S_6$: 2086.9641 found: 2086.9637.
HPLC analysis: t_R = 1.31 min (Chromolith, gradient: 25-45% B/A over 5 min).
HPLC purification: Nucleosil, gradient: 20% B/A over 15 min then 20-35% B/A over 15 min.
Yield: 84%.



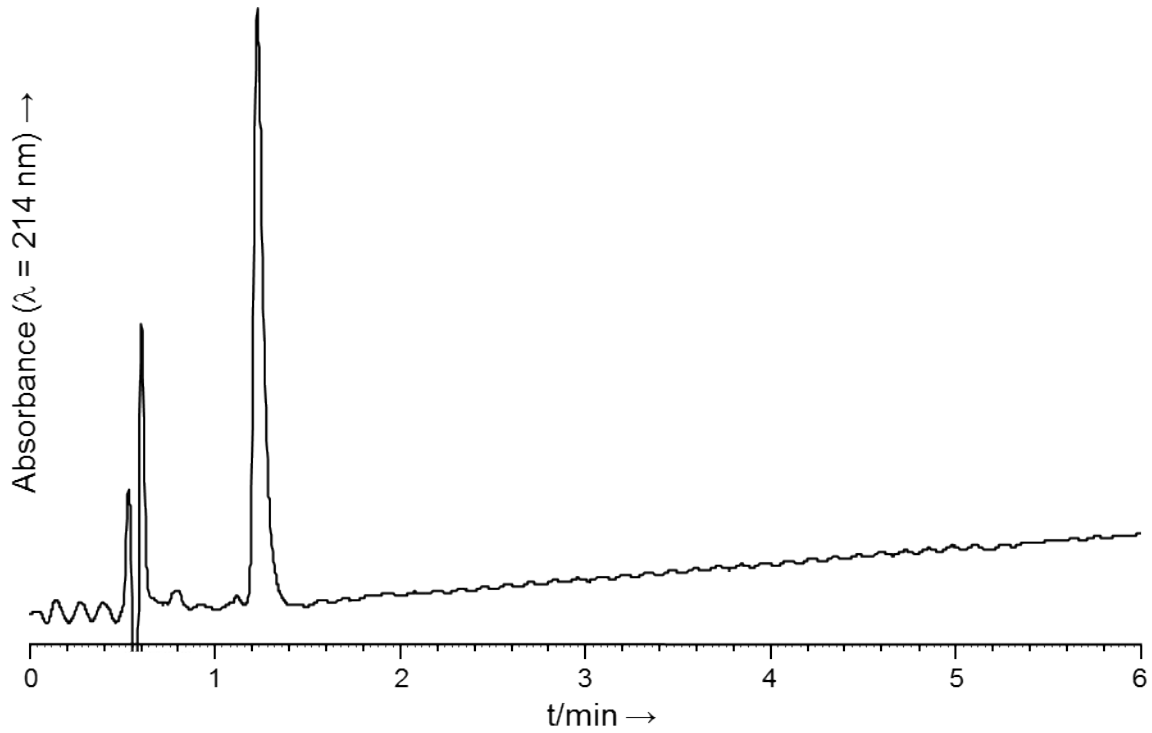
Supplementary figure S29: HPLC trace of crude **4b** after work-up.



Supplementary figure S30: LC/MS analysis of crude **4b** after work-up. Blue trace: UV (λ = 214 nm); red trace: base peak ion chromatogram.

Peak number (t_R (min))	$[MH]^+$ (*m/z*) calcd.	$[MH]^+$ (*m/z*) found	Attributed to
2 / 3 / 4 (5.91-6.07)	2086.9641	2086.9637	**4b**

Supplementary table S12: Attribution of the main peaks observed during LC/MS analysis of crude **4b** after work-up.

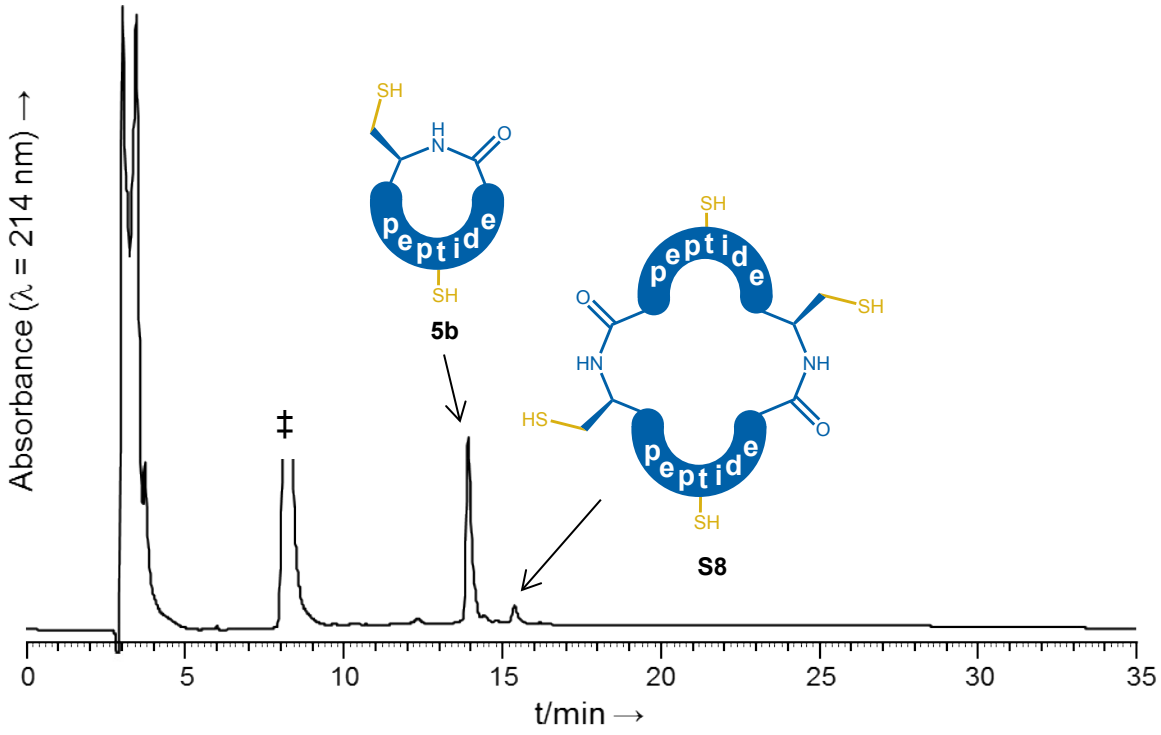


Supplementary figure S31: HPLC trace of purified **4b**.

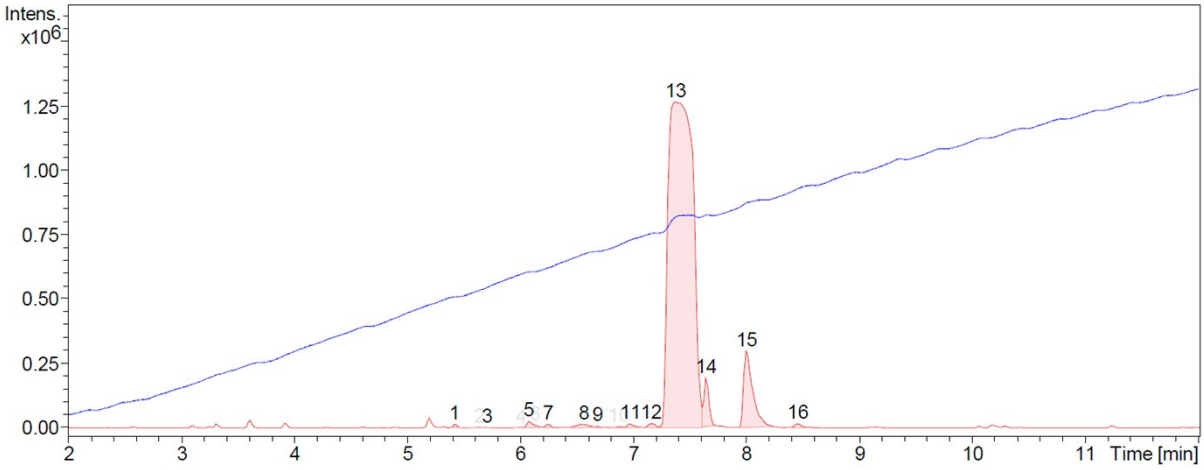
Reduced form of SFTI-1 (**5b**):

Sequence: cyclo(CTKSIPPICFPDGR).

ESI-HRMS (*m/z*): $[MH]^+$ calcd. for $C_{67}H_{107}N_{18}O_{18}S_2$: 1515.7452 found: 1515.7450.
HPLC analysis: t_R = 2.73-2.99 min (Chromolith, gradient: 20-70% B/A over 5 min). t_R = 13.96 min (Nucleosil, gradient: 20-70% B/A over 30 min, 70 °C). HPLC analysis was performed at high temperature due to the presence of large peaks when analysing **5b** on a chromolith column at room temperature, probably due to an equilibrium between two conformers of **5b**.
HPLC purification: Nucleosil, gradient: 25% B/A over 15 min then 25-35% B/A over 10 min., 70°C.
Yield: 79%.



Supplementary figure S32: HPLC trace of crude **5b** (NCL at 1 mM) after work-up (‡: MPAA) (Nucleosil, gradient: 20-70% B/A over 30 min, 70 °C).

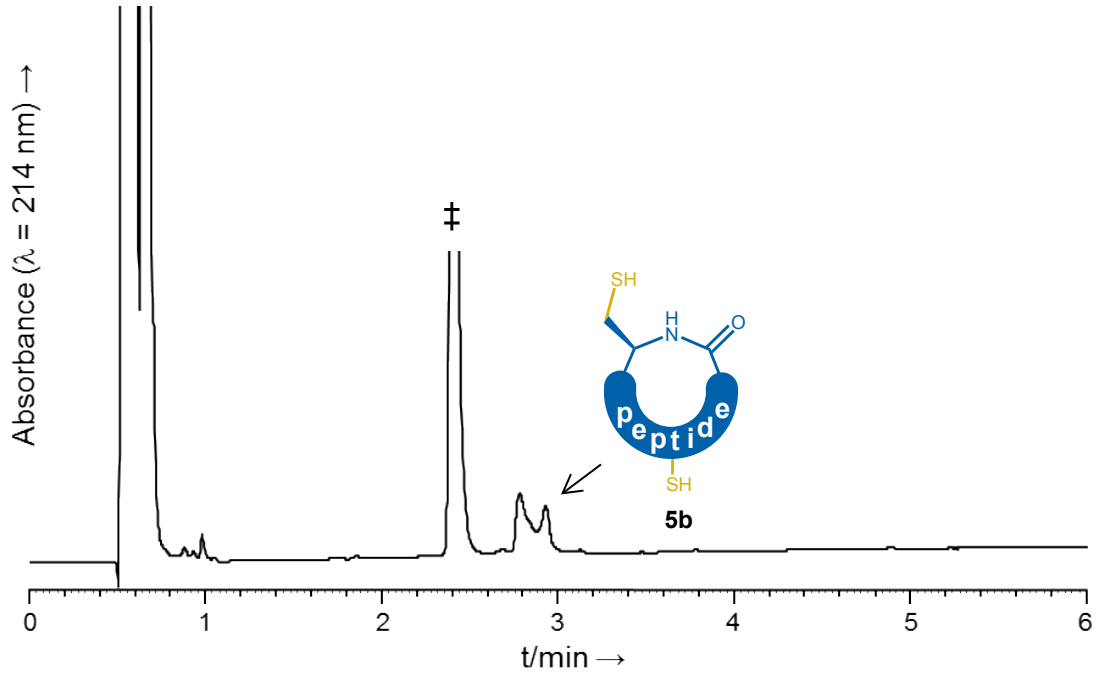


Supplementary figure S33: LC/MS analysis of crude **5b** (NCL at 1 mM) after work-up. Blue trace: UV (λ = 214 nm); red trace: base peak ion chromatogram.

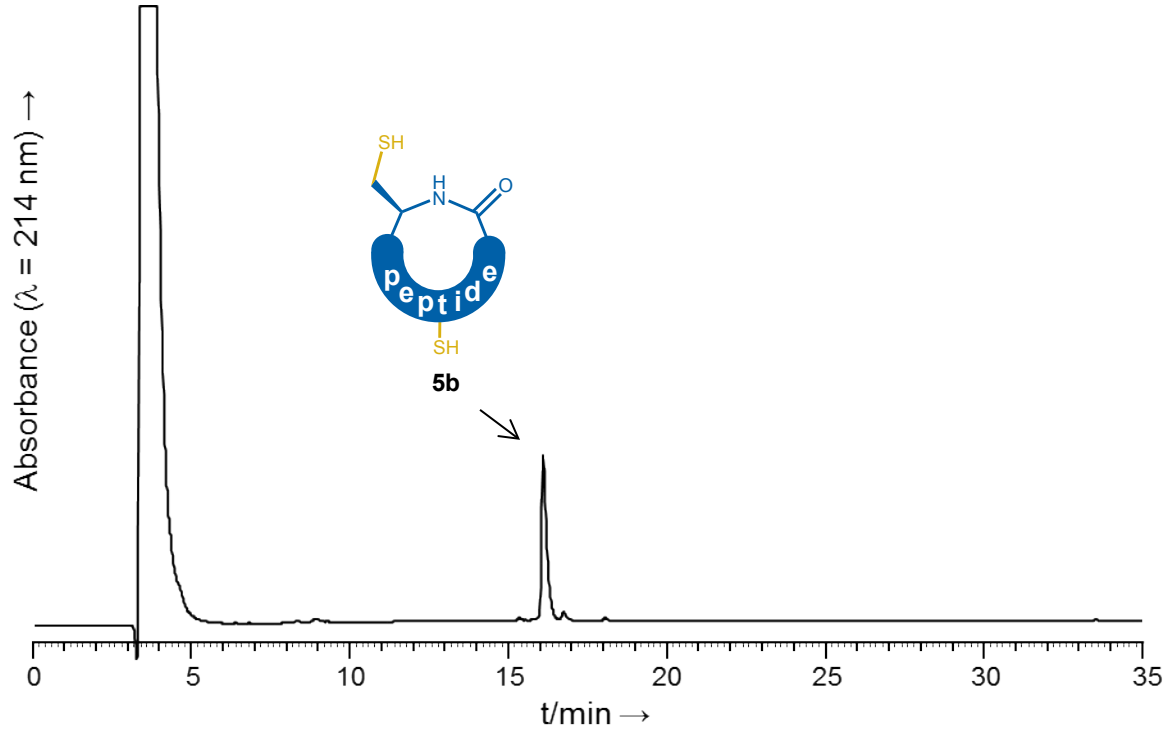
Peak number (t_R (min))	$[MH]^+$ (*m/z*) calcd.	$[MH]^+$ (*m/z*) found	Attributed to
13 (7.37)	1515.7452	1515.7450	**5b**
14 (7.64)	1497.7436	1497.7348	Aspartimide derivative of **5b**
15 (8.00)	3030.4825	3030.4858	Cyclic dimer **S8**[a]

Supplementary table S13: Attribution of the main peaks observed in LC/MS analysis of crude **5b** (NCL at 1 mM) after work-up.

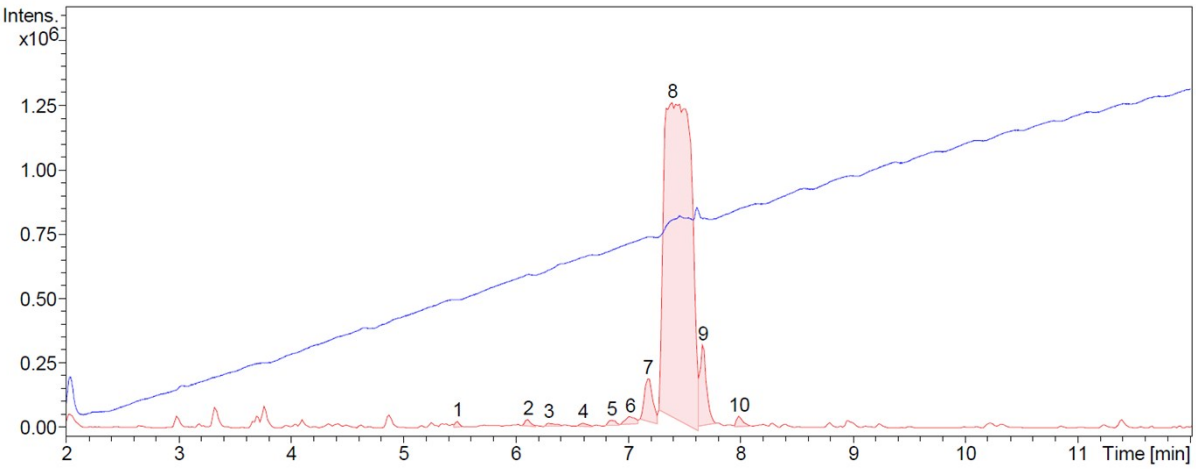
[a]: **S8** = cyclo(CTKSIPPICFPDGRCTKSIPPICFPDGR)



Supplementary figure S34: HPLC trace of crude **5b** (NCL at 0.1 mM) after work-up (‡: MPAA) (Chromolith, room temperature, gradient: 20-70% B/A over 5 min). Note that under these analytical conditions **5b** is eluted as a large double peak probably corresponding to conformers in equilibrium



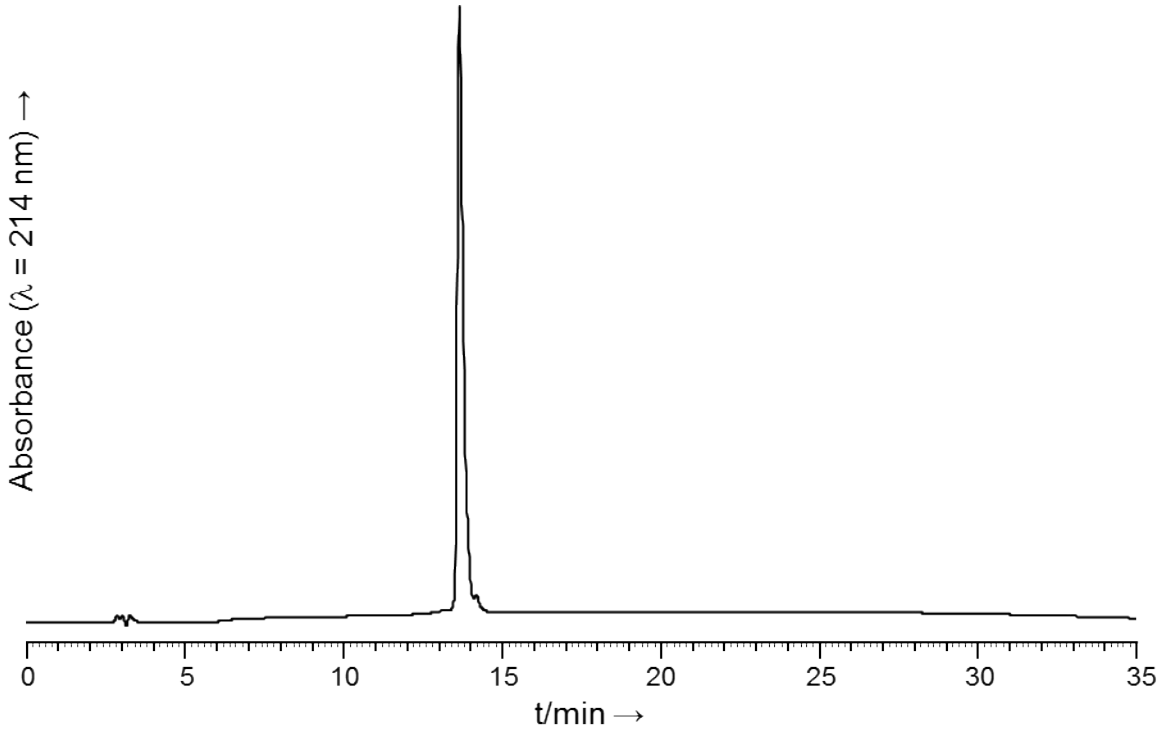
Supplementary figure S35: HPLC trace of crude **5b** (NCL at 0.1 mM) after work-up (Nucleosil, gradient: 20-60% B/A over 30 min, 70 °C).



Supplementary figure S36: LC/MS analysis of crude **5b** (NCL at 0.1 mM) after work-up. Blue trace: UV (λ = 214 nm); red trace: base peak chromatogram.

Peak number (t_R (min))	$[MH]^+$ (*m/z*) calcd.	$[MH]^+$ (*m/z*) found	Attributed to
8 (7.39)	1515.7452	1515.7440	**5b**
9 (7.66)	1497.7436	1497.7350	Aspartimide derivative of **5b**
10 (7.98)	3030.4825	3030.4827	Cyclic dimer **S8**

Supplementary table S14: Attribution of the main peaks observed in LC/MS analysis of crude **5b** (NCL at 0.1 mM) after work-up.



Supplementary figure S37: HPLC trace of purified **5b**.