Supplementary Information

Direct synthesis of cyclic lipopeptides using intramolecular native chemical ligation and thiol-ene CLipPA chemistry

Victor V. Yim,[a,b] Iman Kavianinia,[b,c] Alan J. Cameron,[b,c] Paul W. R. Harris,*[b,c] and Margaret A. Brimble*[a,b,c]

[a]*School of Chemical Sciences, The University of Auckland, 23 Symonds St., Auckland 1010, New Zealand*

[b]*School of Biological Sciences, The University of Auckland, 3A Symonds St., Auckland 1010, New Zealand*

[c]*Maurice Wilkins Centre for Molecular Biodiscovery, The University of Auckland, 3A Symonds St., Auckland 1010, New Zealand*

**Email: m.brimble@auckland.ac.nz*

Table of Contents

S1. Abbreviations

°C: degrees celcius. 2-BrZ: 2-bromobenzyloxycarbonyl. 6-Cl-HOBt: 6-chloro-1-hydroxybenzotriazole. A = ampere (s). Ar: argon. Asn: asparagine. Boc: *tert*-butyloxycarbonyl. Boc_2O: di-*tert*-butyl-dicarbonate. Bzl: benzyl. *ca.*: *circa*/approximately. CLipPA: Cysteine Lipidation on a Peptide or Amino acid. 2-CTC: 2-chlorotrityl chloride. Cys: cysteine. Dbz: diaminobenzoic acid. DIC: *N,N'*-diisopropylcarbodiimide. DIPEA: *N,N*-diisopropylethylamine. DMF: *N,N*-dimethylformamide. DMPA: 2,2-dimethoxy-2-phenylacetophenone. DODT: 2,2'-(ethylenedioxy)-diethanethiol. eq.: molar equivalence. ESI-MS: electrospray ionisation mass spectrometry. Fmoc: (9*H*-fluoren-9-yl)methoxycarbonyl. Gln: glutamine. Gly: glycine. h: hour(s). HATU: *O*-(7-azabenzo-triazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate. HBTU: *O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate. HPLC: high performance liquid chromatography. Hz: Hertz. hv: light. LC: liquid chromatography. LC-MS: liquid chromatography-mass spectrometry. M: molar. mg: milligram. min: minute (s). mL: millilitres. mmol: millimoles. MPAA: 4-mercaptophenylacetic acid. MQ: Milli-Q. MS: mass spectrometry. *m/z*: mass per charge. NCL: native chemical ligation. nm: nanometre. NMP: *N*-methyl-2-pyrrolidone. *p*: *para*. PAM: 2-(4-(hydroxymethyl)phenyl)acetic acid. Pro: proline. r.t.: room temperature. Rink: 4-((2,4-dimethoxyphenyl)aminomethyl)phenoxyacetic acid. RP: reverse phase. t_R: retention time. Ser: serine. SPPS: solid-phase peptide synthesis. *t*Bu: *tert*-butyl. *t*Non: *tert*-nonyl. TCEP: tris(2-carboxylethyl)phosphine. TFA: trifluoroacetic acid. Thz: thiazolidine. TIPS: triisopropylsilane. *t*NonSH: *tert*-nonyl mercaptan. Trt: triphenylmethyl. Tyr: tyrosine. UV: ultraviolet. V: volts. *v/v*: volume per volume. W: Watt (s). Xan: Xanthenyl. λ: lambda/wavelength. µL: microliter. µm: micrometre. µwave: microwave.

S2. General Information

All reagents were purchased as reagent grade and used without further purification unless otherwise noted. 6-Cl-HOBt and Fmoc-D-Asn (Trt)-OH were purchased from AAPPTec (Louisville, KY). Fmoc-Rink amide linker and Fmoc-D-Cys(Trt)-OH was purchased from AK Scientific (Union City, CA). Boc-Gln-OH was purchased from Auspep (Tullamarine, Australia). Argon was purchased from BOC group (Guildford, UK). Boc-D-Thz-OH was purchased from Chem Impex (Wood Dale, IL). 2CTC resin and Fmoc-D-Tyr(*t*Bu)-OH were purchased from ChemPep (Wellington, FL). CH_2Cl_2, diethyl ether, HCl, MeCN, MeOH and Na_2HPO_4 were purchased from ECP (Auckland, New Zealand). Boc_2O, Fmoc-Asn(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Pro-OH, Fmoc-Ser(*t*Bu)-OH, HATU and HBTU were purchased from GL Biochem (Shanghai, China). Anhydrous HF was purchased from Matheson Trigas (Basking Ridge, NJ). TFA was purchased from Oakwood Chemicals (Estill, NC). Boc-D-Asn(Xan) was purchased from Peptides International (Louisville, KY). Boc-D-Tyr(2Br-Z)-OH, Boc-Gly-PAM linker, Boc-Pro-OH, Boc-Ser(Bzl)-OH) were purchased from Polypeptides (Strasbourg, France). Aminomethyl-polystyrene resin was purchased from Rapp polymere (Tübingen, Germany). DMF and NaOH pellets were purchased from Scharlau (Barcelona, Spain). DIC, Dbz-OH (mono Fmoc protected before use), DIPEA, DMPA, DODT, iturin A, methoxyamine.HCl, 3-MPA, MPAA, NMP, *p*-cresol, piperidine, TCEP.HCl, TIPS, *t*NonSH, vinyl benzoate, vinyl butyrate, vinyl decanoate, vinyl pivalate, vinyl 4-*tert*-butylbenzoate were

purchased from Sigma-Aldrich (St. Louis, MO). Vinyl palmitate was purchased from TCI (Tokyo, Japan). H_2O was purified using a Sartorius (Göttingen, Germany) arium® pro ultrapure water system.

Microwave assisted reactions were performed in a CEM (Matthews, NC) Discover SP, Model 908010 reactor. Analytical RP-HPLC was used to analyse final compounds and performed on a Dionex (Sunnyvale, CA) UltiMate 3000 system using a Waters (Milford, MA) Xterra MS C18 (5 µm 4.6 × 150 mm) column, and Chromeleon software was used for data processing. Buffer A: 0.1% (*v/v*) TFA in H_2O; buffer B: 0.1% (*v/v*) TFA in MeCN. LC-MS was performed on an Agilent (Santa Clara, CA) 1260 Infinity equipped with an Agilent 6120 Quadrupole LC-MS using an Agilent Zorbax 300SB-C3 column (3.5 µm, 3.0 × 150 mm). Data processing was carried out by Agilent OpenLAB software. Buffer A: 0.1% (*v/v*) formic acid in H_2O; buffer B: 0.1% (*v/v*) formic acid in MeCN. Crude peptides was purified on Dionex UltiMate 3000 preparative HPLC using Agilent Zorbax 300SB-C18 column (5 µm, 9.4 × 250 mm) and Chromeleon software was used for data processing. Ultraviolet irradiation was carried out using Spectroline (Westbury, NY) hand-held lamp EA-160/FA, 6 W integrally filtered tube at 50 Hz, 0.17 A and λ = 365 nm.

Biological assays were performed inside a Thermo Fisher (Waltham, MA) HERAsafe KSP 12 biological safety cabinet on 96-well plates obtained from MediRay (Auckland, New Zealand) with *Candida albicans* SC5314. Roswell Park Memorial Institute 1640 (RPMI 1640) media was obtained from Thermo Fisher (Waltham, MA). Media and fungi were incubated in an N-Biotek (Geyonggi, South Korea) NB205L shaker.

S3. General Methods

General Method A: loading the linker

To pre-swollen (CH_2Cl_2/DMF (1:1 *v/v*)) aminomethyl-polystyrene resin (110 mg, 0.1 mmol, loading 0.91 mmol/g) was attached the relevant linker. The completion of the coupling was monitored using the ninhydrin test.[1] If the coupling was incomplete, the coupling procedure was repeated with freshly prepared reagents.

General Method B: Fmoc-SPPS under microwave irradiation

N$^{\alpha}$-Fmoc removal was achieved with piperidine/DMF (1:4 *v/v*) under microwave irradiation (50 W, 75 °C, 2 × 3 min), *N*$^{\alpha}$-protected amino acids (0.4 mmol, 4 eq.) were coupled onto the free amino group with HATU (144 mg, 0.38 mmol, 3.8 eq.) and DIPEA (139 µL, 0.8 mmol, 8 eq.) in DMF under microwave irradiation (25 W, 50 °C, 5 min). Deprotection and coupling steps were repeated for each amino acid until the completed linear resin-bound peptide sequence was afforded. To confirm the identity of the assembled linear sequence, a small fraction of the resin was treated with TFA/DODT/H_2O/TIPS (94:2.5:2.5:1 *v/v/v/v*) to release the peptide for analysis by LC-MS.

General Method C: NCL cyclisation

An NCL buffer was prepared with 0.2 M Na_2HPO_4, 0.1 M TCEP·HCl and 0.02 M MPAA in MQ H_2O with pH adjusted to 7.5 and sparged the solution with Ar for 15 min. The linear peptide precursor was dissolved in the buffer at 10 mM peptide concentration and shaken for 1 h at r.t. Cyclisation progress was monitored by taking 20 µL aliquots, acidified by an equal volume of aqueous HCl (5 M) and analysed with LC-MS using an Agilent Zorbax 300SB-C3 column (3.5 µm, 3.0 × 150 mm) and a linear gradient of 5% B to 65% B over 20 min, *ca.* 3% B per minute at r.t., 0.3 mL/min.

General Method D: Boc-SPPS

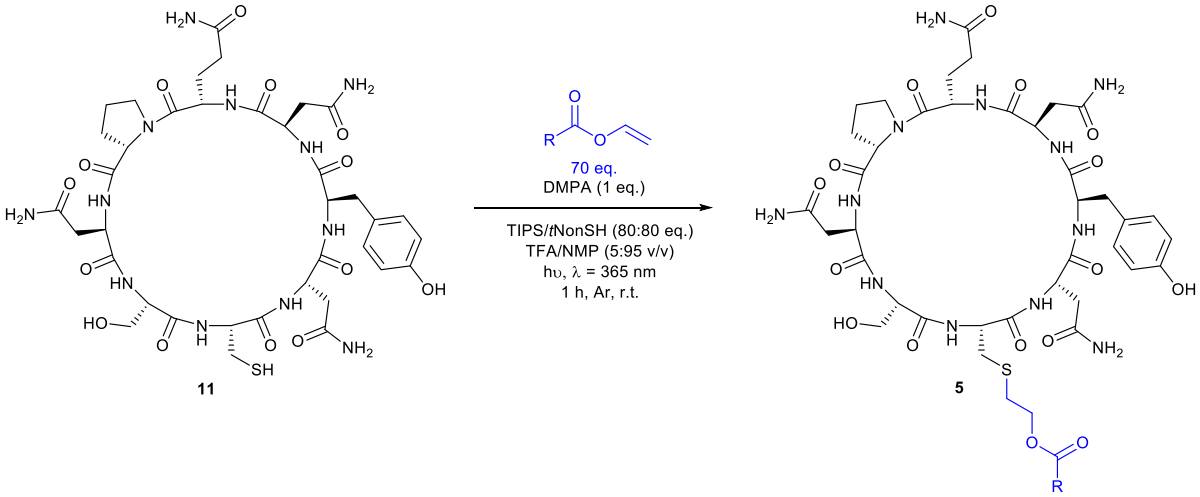
N$^{\alpha}$-Boc removal was achieved by treating the peptidyl resin with neat TFA at r.t. (2 × 1 min) followed by DMF flow wash (3 × 30 s). If the residue is Gln, the resin was washed with CH_2Cl_2 before *N*$^{\alpha}$-Boc deprotection to prevent pyrrolidone side-product from the heat of TFA-DMF interactions.[2] *N*$^{\alpha}$-Boc protected amino acids (0.4 mmol, 4 eq.) were coupled onto the free amino group with HATU (144 mg, 0.38 mmol, 3.8 eq.) and DIPEA (139 µL, 0.8 mmol, 8 eq.) in DMF at r.t. (5 min). Deprotection and coupling steps were repeated until the completed linear resin-bound peptide sequence was afforded.

General Method E: purification

Crude peptide was dissolved in 0.1% (*v/v*) TFA in H_2O:MeCN (4:1 *v/v*), centrifuged and filtered using Phenomenex (Torrance, CA) Phenex syringe filters (26 mm, 0.45 µm). The filtrate was injected in 2500 µL aliquots and purified using a slow gradient on RP-HPLC (1 – 95% B, 1% B/min, 4 mL/min).[3] The fractions were collected based on UV absorbance at wavelengths of 210 nm, 230 nm, 254 nm and 280

nm, followed mass-spectrometry analysis (ESI^+ 100 V; H_2O:MeCN; 1:1 *v/v*, 0.2 mL/min). The fractions containing the purified peptide were combined and lyophilised.

General Method F: conjugating vinyl esters onto cyclic scaffold 11



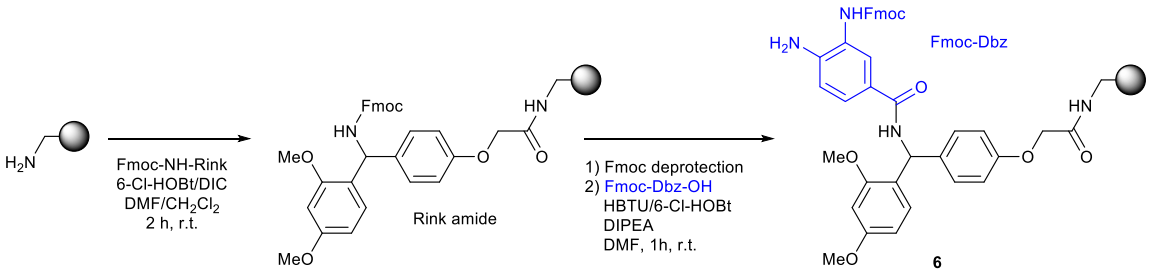
Cyclic peptide **11** was dissolved in NMP (10 mg/mL), along with the desired vinyl ester (70 eq.), DMPA (1 eq.) and TIPS (80 eq.), the mixture was then degassed with argon for 15 min. TFA (5% *v/v*) and *t*NonSH (80 eq.) were then added to the mixture under argon and the mixture irradiated under at 365 nm, r.t., 1 h. The reaction was monitored using LC-MS (5 – 95% B, 3% B/min, 0.3 mL/min). Upon completion of the reaction, the mixture was triturated with diethyl ether (40 mL, 4 °C). Centrifuging gave a peptide pellet which was dissolved in 0.1% (*v/v*) TFA in H_2O:MeCN (1:1). Purification according to **General Method E** gave purified peptide **5**.

S4. Experimental Section

S4.1. Fmoc approach for the synthesis of cyclic peptide 11

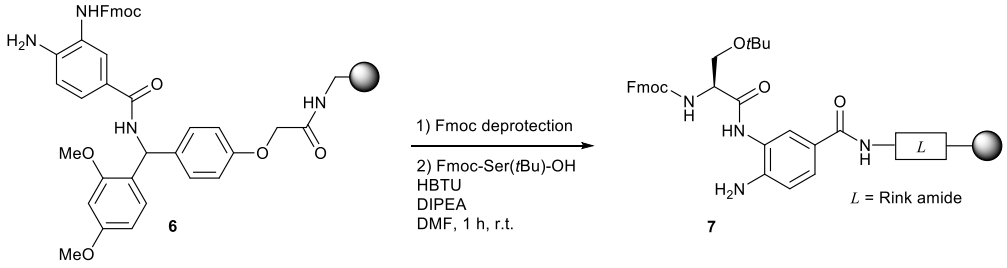
S4.1.1. *N*-acyl-benzimidazolinone thioester precursor

i) Attaching the linkers to aminomethyl-polystyrene resin



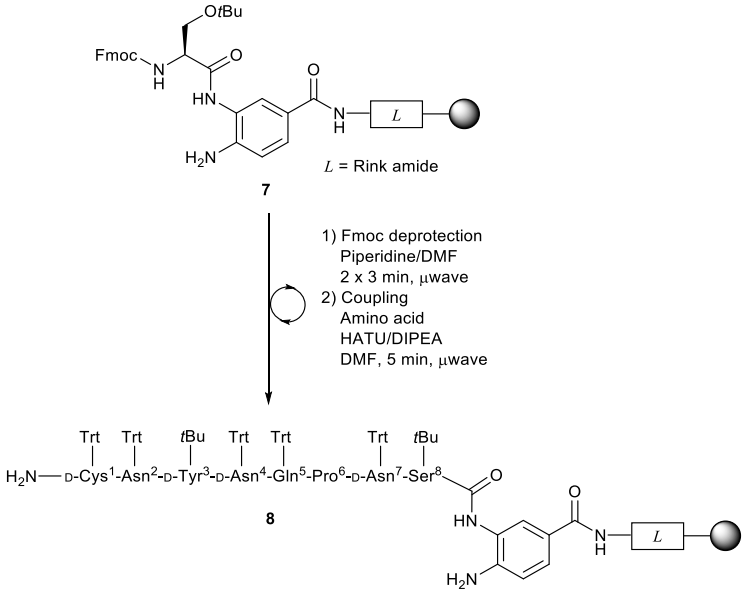
Fmoc-Rink amide linker (4 eq.) was attached to aminomethyl-polystyrene resin (110 mg, 0.1 mmol, loading 0.91 mmol/g) using 6-Cl-HOBt (68 mg, 0.4 mmol, 4 eq.) and DIC (62.6 µL, 0.4 mmol, 4 eq.) dissolved in DMF/CH_2Cl_2 (1:4 *v/v*) according to **General Method A**. The Fmoc group was removed as described in **General Method B**. Fmoc-Dbz-OH (150 mg, 0.4 mmol, 4 eq.), HBTU (152 mg, 0.4 mmol, 4 eq.), and 6-Cl-HOBt (68mg, 0.4 mmol, 4 eq.) were dissolved in DMF (3 mL). DIPEA (105 µL, 0.6 mmol, 6 eq.) was added to activate the Dbz linker for 30 s, after which the mixture was added to the Rink amide resin and gently agitated for 1 h at r.t. The solution was then drained, and the resin was washed with DMF (3 × 5 mL) and dried by washing with MeOH (3 × 5 mL) to give linker resin **6**.

ii) Coupling the first amino acid



Fmoc deprotection was achieved according to **General Method B**. Fmoc-Ser(*t*Bu)-OH (230 mg, 0.6 mmol, 6 eq.) and HBTU (228 mg, 0.6 mmol , 6 eq.) were dissolved in DMF (3 mL), and DIPEA (157 µL, 0.9 mmol, 9 eq.) was added to activate the amino acid. After 1 min, the mixture was added to resin **6**. The solution was then drained, and the resin was washed with DMF (3 × 5 mL) and dried by washing with MeOH (3 × 5 mL) to give peptidyl resin **7**.

*iii) Microwave enhanced elongation to generate linear peptidyl resin **8***



Resin **7** was elongated with Fmoc-AA-OH (0.4 mmol, 4 eq.) under microwave assistance according to **General Method B**. Starting with Fmoc-D-Asn(Trt)-OH (239 mg), Fmoc-Pro-OH (135 mg), Fmoc-Gln(Trt)-OH (244 mg), Fmoc-D-Asn(Trt)-OH (239 mg), Fmoc-D-Tyr(*t*Bu)-OH (184 mg), Fmoc- Asn(Trt)-OH (239 mg) and Fmoc-D-Cys(Trt)-OH (234 mg) were sequentially coupled onto **7**, followed by N^{α}-Fmoc deprotection after each coupling to afford peptidyl resin **8**, the identity was confirmed by analysing cleaved and deprotected sample **SI 1** according to **General Method B** using LC-MS: t_R = 18.1 min, $[M + H]^+$ found 1294.4, $[C_{59}H_{71}N_{15}O_{17}S + H]^+$ requires 1294.5 (**Figure S1**), by-product **12** was identified as the hydrolysis of the Dbz linker during deprotection: t_R = 10.3 min, $[M + H]^+$ found 938.5, $[C_{37}H_{55}N_{13}O_{14}S + H]^+$ requires 938.4.

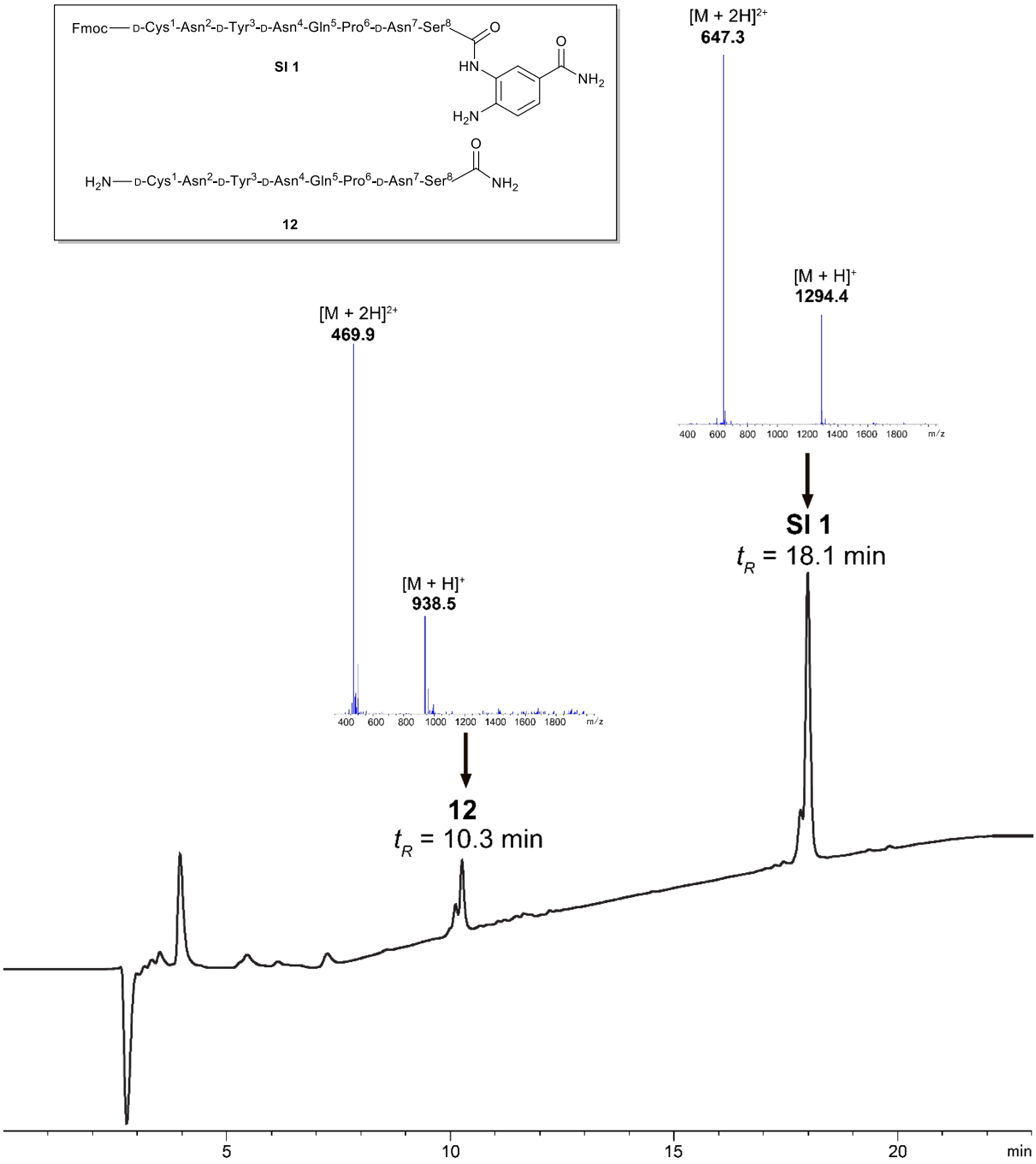
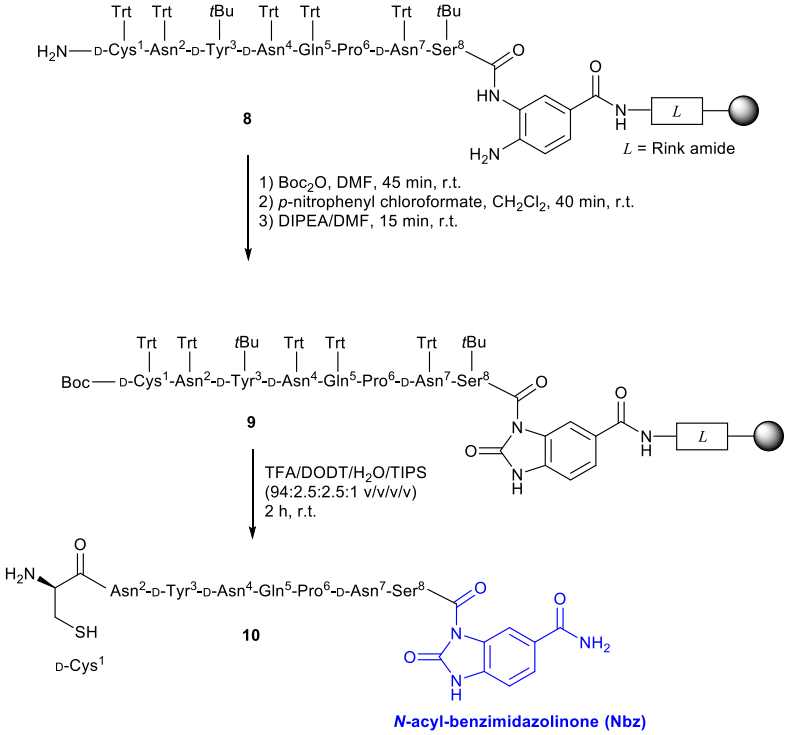


Figure S1. LC-MS analysis of cleavage product of intermediate peptide **SI 1**. **12** is the truncated form of **SI 1** as a result of incomplete Dbz linker coupling. Structures of **SI 1** and **12** shown (top, boxed). LC-MS was performed with Agilent Zorbax 300SB-C3 (3.5 µm, 3.0 × 150 mm), linear gradient of 5% B to 65% B over 20 min, *ca.* 3% B per minute at r.t., 0.3 mL/min.

iv) Generating cyclic precursor with C-terminal Nbz leaving group ***10***



Boc protection on N^{α}-terminal amine was achieved through gentle agitation of resin **8** with Boc_2O (218 mg, 1.0 mmol, 10 eq.) in DMF (3 mL) for 45 min at r.t. The solution was drained and a negative ninhydrin test result confirmed N^{α}-Boc protection on D-Cys. The resin was then swollen in CH_2Cl_2 for 15 min. After addition of *p*-nitrophenyl chloroformate (101 mg, 0.5 mmol, 5 eq.) in CH_2Cl_2 (3 mL), the resin was gently agitated for 40 min at r.t., the solvent drained and the resin washed with DMF (3 × 5 mL) and DIPEA (261 µL, 1.5 mmol, 0.5 M in DMF, 3 mL) added, producing a yellow colouration. After 15 min of gentle agitation, the solvent was drained, the resin washed with DMF (3 × 5 mL), then MeOH (3 × 5 mL) and dried to give peptidyl-Nbz-resin **9.** The peptide was released from the resin by treating with TFA/DODT/H_2O/TIPS (94:2.5:2.5:1, *v/v/v/v*), to give crude cyclic precursor **10** (75 mg, 70% yield,66% purity) as confirmed by LC-MS: t_R = 10.7 min, $[M + H]^+$ found 1098.5, $[C_{45}H_{59}N_{15}O_{16}S + H]^+$ requires 1098.4 (**Figure S2**).

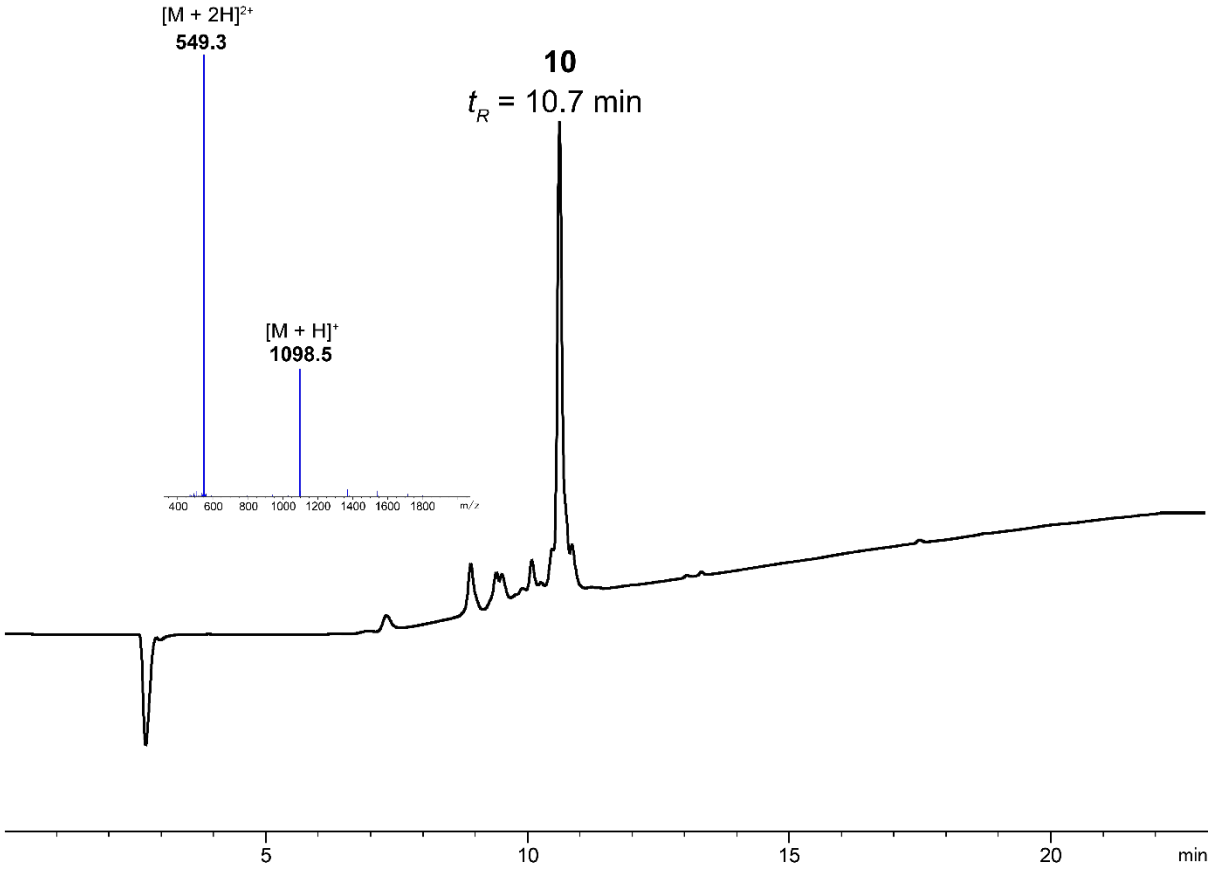
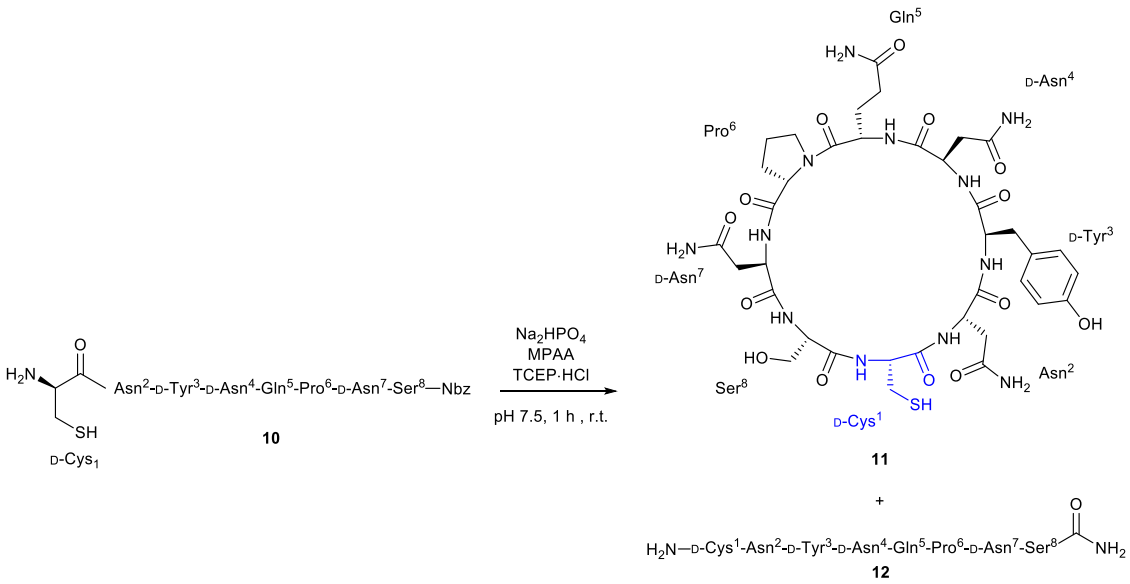


Figure S2. LC-MS analysis of crude peptide **10**. LC-MS was performed with Agilent Zorbax 300SB-C3 (3.5 µm, 3.0 × 150 mm), linear gradient of 5% B to 65% B over 20 min, *ca.* 3% B per minute at r.t., 0.3 mL/min.

*v) Cyclisation of peptide **11** effected by NCL*



Crude peptide **10** was dissolved in the NCL buffer with thiol catalyst MPAA and cyclised according to **General Method C** to give **11** in 23% conversion as confirmed by LC-MS: t_R = 10.1 min, $[M + H]^+$ found 921.3, $[C_{37}H_{52}N_{12}O_{14}S + H]^+$ requires 921.3, and 77% conversion to by product **12**: t_R = 6.6 min, $[M + H]^+$ found 938.5, $[C_{37}H_{55}N_{13}O_{14}S + H]^+$ requires 938.4 (**Figure S3**).

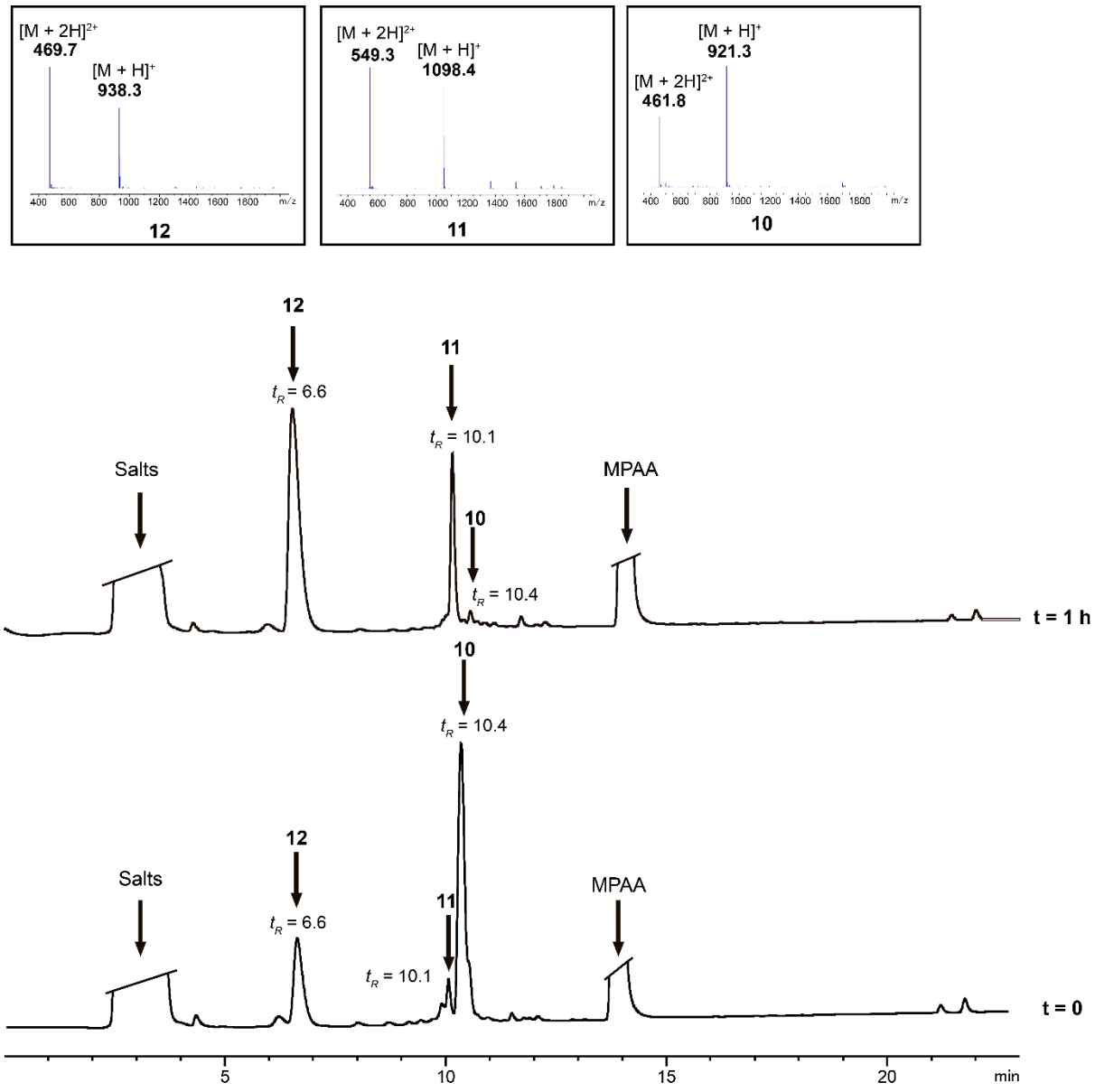
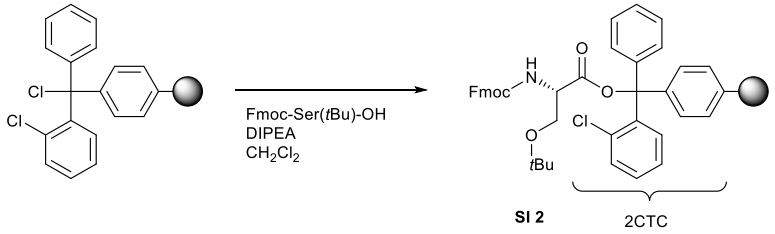


Figure S3. LC-MS analysis of cyclisation of **10** with NCL after 1 h. LC-MS was performed with Agilent Zorbax 300SB-C3 (3.5 µm, 3.0 × 150 mm), linear gradient of 5% B to 65% B over 20 min, *ca.* 3% B per minute at r.t., 0.3 mL/min.

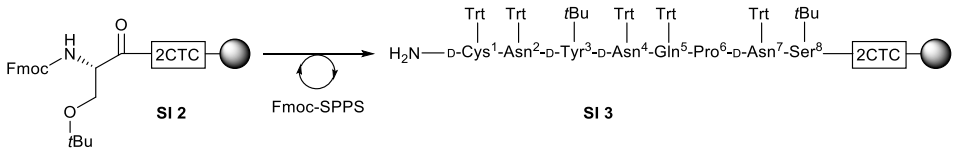
S4.1.2. Solution-phase direct thioesterification

i) Loading the first amino acid



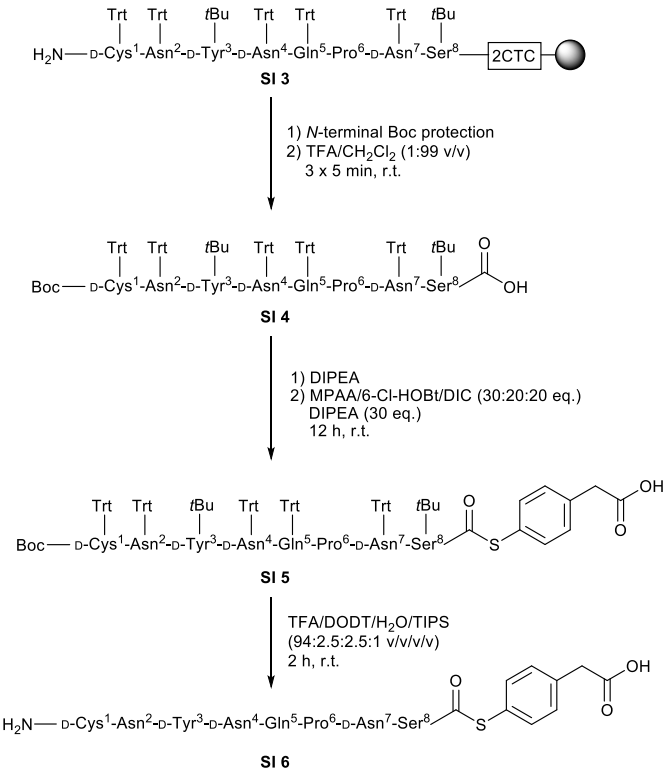
To pre-swollen (CH_2Cl_2) 2-chlorotrityl chloride resin (130 mg, 0.1 mmol, 0.77 mmol/g) was added Fmoc-Ser(*t*Bu)-OH (153 mg, 0.4 mmol, 4 eq.) and DIPEA (174 µL, 1.0 mmol, 10 eq.) in CH_2Cl_2 (3 mL) and gently agitated for 1 h at r.t to give **SI 2**. The loading was determined to be 62% quantified by an Fmoc release assay using a fraction of the resin (ca. 1 mg).[4] Unreacted sites were capped by treating with CH_2Cl_2/MeOH/DIPEA (8:1.5:0.5 *v/v/v*, 2 × 10 min, r.t.).

ii) Elongating the peptide with Fmoc-SPPS



The peptide was then elongated following manual Fmoc-SPPS procedures adapted from **General Method B** using Fmoc-AA-OH (0.5 mmol, 5 eq.), HATU (183 mg, 0.48 mmol, 4.8 eq.) and DIPEA (174 µL, 1.0 mmol, 10 eq.) in DMF at r.t. (45 min) and deprotection using piperidine/DMF (1:4 *v/v*, 2 × 5 min) to generate peptidyl resin **SI 3**.

iii) In situ direct thioesterification



N-terminal amine of **SI 3** was Boc protected as previously described in **Section S4.1.1 iv)**. The peptide was released by treatment with TFA/CH_2Cl_2 (1:99 *v/v*, 3 mL) 3 × 5 min at r.t.. The solution containing protected peptide **SI 4** was drained into a round bottom flask containing DIPEA (225 µL, 1.29 mmol, 1.1 eq. with respect to TFA). To this solution was added MPAA (323 mg, 35.2 mmol, 30 eq.), 6-Cl-HOBt (211 mg, 23.5 mmol, 20 eq.), DIC (194 µL, 23.5 mmol, 20 eq.) and additional DIPEA (225 µL, 1.29 mmol, 1.1 eq.). The mixture was stirred for 12 h at r.t.. The solution was sparged with nitrogen to evaporate TFA, and the residual CH_2Cl_2 was removed by rotary evaporation at 40 °C. Trituration with diethyl ether (4 °C) gave crude protected thioester **SI 5**. Global deprotection with TFA/DODT/H_2O/TIPS (94:2.5:2.5:1 *v/v/v/v*) gave **SI 6** (12 mg, 11% yield, 70% purity) as confirmed by LC-MS: t_R = 11.1 min, $[M + H]^+$ found 1089.3, $[C_{45}H_{60}N_{12}O_{16}S + H]^+$ requires 1089.4 (**Figure S4**).

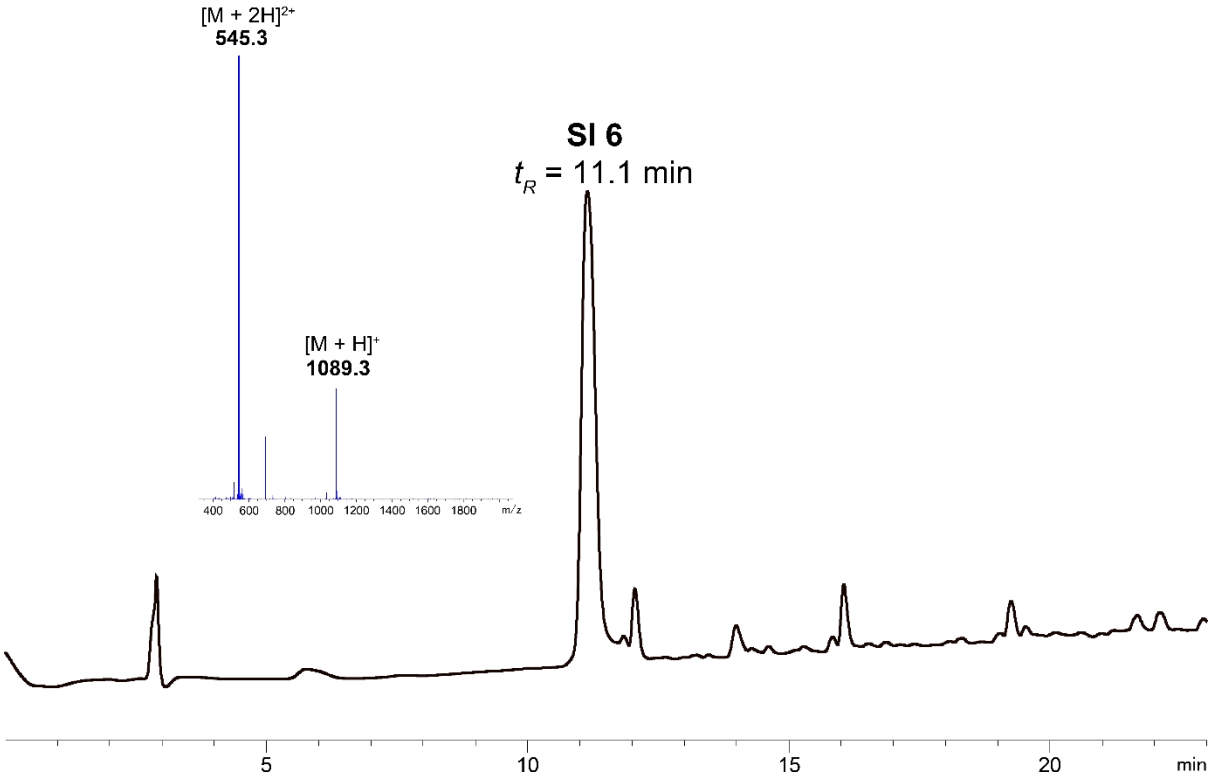
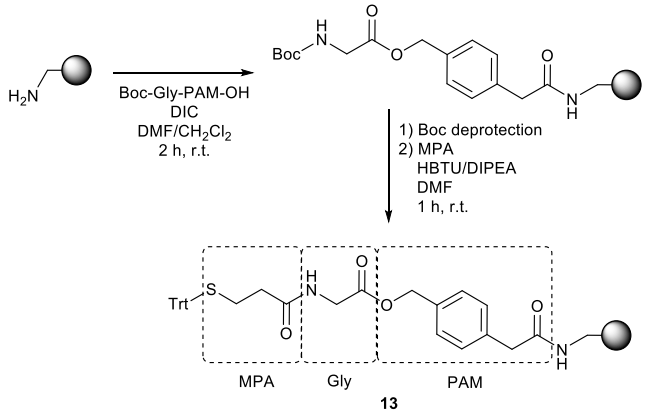


Figure S4. LC-MS analysis of crude peptide **SI 6** after *in situ* direct thioesterification. LC-MS was performed with Agilent Zorbax 300SB-C3 (3.5 µm, 3.0 × 150 mm), linear gradient of 5% B to 65% B over 20 min, *ca.* 3% B per minute at r.t., 0.3 mL/min.

S4.2. Boc-SPPS approach for the synthesis of cyclic peptide 11

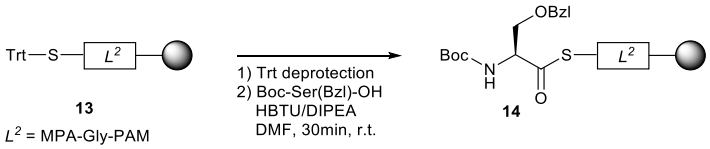
S4.2.1. Forming the thioester on resin

i) Forming the thiol linker on resin



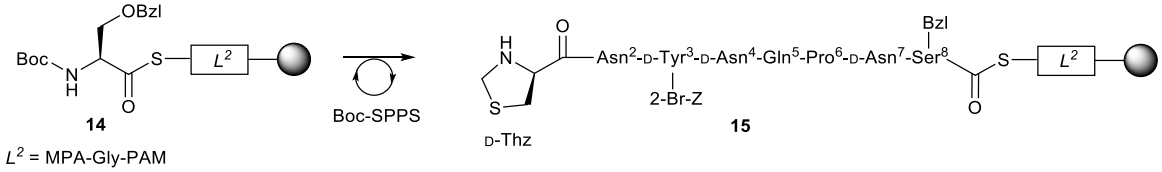
Boc-Gly-PAM linker (129 mg, 0.4 mmol, 4 eq.) was attached onto aminomethyl-polystyrene resin (110 mg, 0.1 mmol, loading 0.91 mmol/g) with DIC (63 µL 0.4 mmol, 4 eq.) according to **General Method A**. The Boc group was removed as described in **General Method D** using neat TFA. 3-(Trityl)mercaptopropionic acid (139 mg, 0.4 mmol, 4 eq.) and HBTU (144 mg, 0.38 mmol, 3.8 eq.) were dissolved in DMF (3 mL). DIPEA (70 µL, 0.4 mmol, 4 eq.) was added to activate the acid for 30 s, after which the mixture was added to the H_2N-Gly-PAM resin and gently agitated for 30 min at r.t. The solution was then drained, and the resin washed with DMF (3 × 5 mL) to give Trt-protected thiol-linker-resin **13**.

ii) Generating the C-terminal thioester



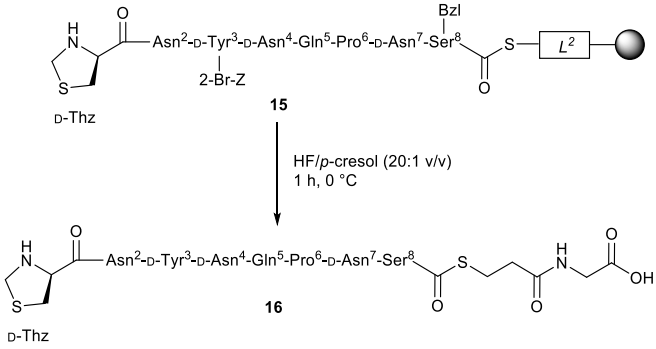
S-Trt deprotection was carried out by treating **13** with TFA/DODT/H_2O/TIPS (94:2.5:2.5:1 *v/v/v/v*) with gentle agitation for 2 × 2 min at r.t. The solution was drained and the resin was flow washed with DMF (3 × 30 s). Boc-Ser(Bzl)-OH (78 mg, 0.4 mmol, 4 eq.) and HBTU (144 mg, 0.38 mmol, 3.8 eq.) were dissolved in DMF. DIPEA (70 µL, 0.4 mmol, 4 eq.) was added to activate Boc-Ser(Bzl)-OH for 30 s, after which the mixture was added to thiol-linker-resin and gently agitated for 30 min at r.t. to generate thioester-resin **14**.

iii) Elongating the sequence with Boc-SPPS



The remaining sequence was elongated using Boc-SPPS following conditions described in **General Method D**. Starting with Boc-D-Asn(Xan)-OH, Boc-Pro-OH, Boc-Gln-OH, Boc-D-Asn(Xan)-OH, Boc-D-Tyr(2Br-Z)-OH, Boc-Asn(Xan)-OH and Boc-D-Thz-OH were sequentially coupled onto **14**, followed by N^{α}-Boc deprotection after each coupling to afford peptidyl-thioester-resin **15**.

iv) Releasing the peptide from solid support



The thioester-peptide was concomitantly deprotected and released from the resin by treatment with HF/*p*-cresol (20:1, *v/v*, 10 mL), stirring for 1 h at 0 °C. HF was then slowly evaporated with a vacuum generated by a water aspirator and the residue was triturated with diethyl ether (4 °C) and centrifuged.[5] The peptide pellet was dissolved in 0.1% TFA (*v/v*) in H_2O:MeCN (1:1 *v/v*) and lyophilised to give crude peptide **16** as a fluffy white powder (73mg, 67% yield, 53% purity) as confirmed by LC-MS: t_R = 8.7 min, $[M + H]^+$ found 1096.3, $[C_{43}H_{61}N_{13}O_{17}S + H]^+$ requires 1096.4 (**Figure S5**).

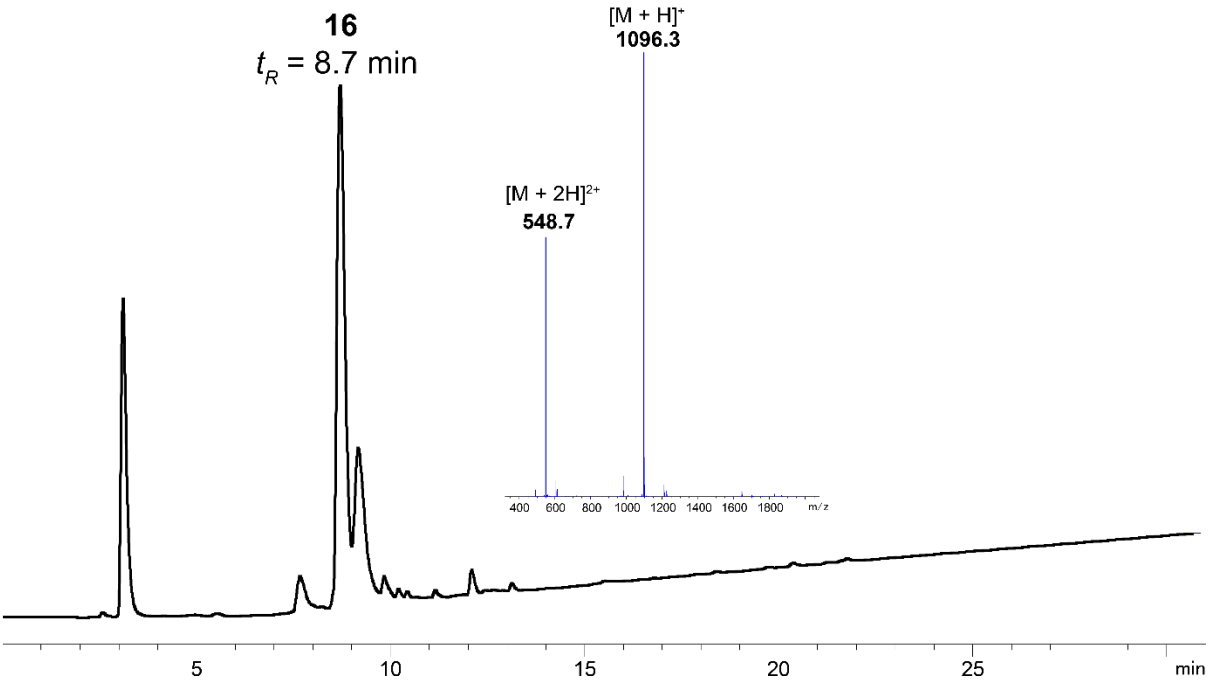
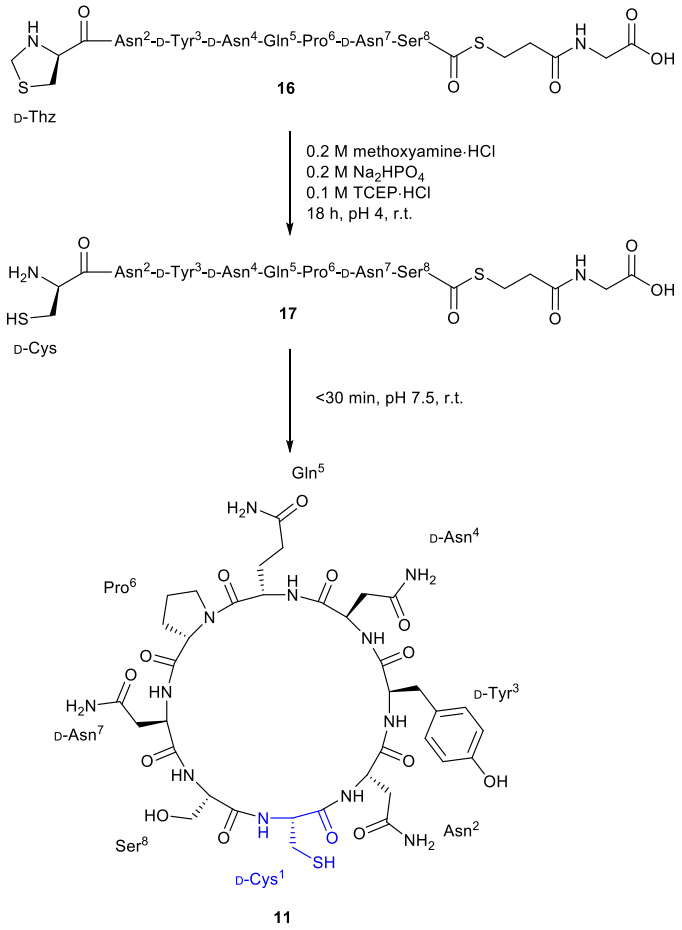


Figure S5. LC-MS analysis of crude peptide **16** with *N*-terminal Thz. LC-MS was performed with Agilent Zorbax 300SB-C3 (3.5 µm, 3.0 × 150 mm), linear gradient of 5% B to 95% B over 30 min, *ca.* 3% B per minute at r.t., 0.3 mL/min.

v) In situ unmasking of thiazolidine and cyclisation effected by NCL



Crude thioester **16** (73 mg) was dissolved to a concentration of 50 mM in a buffer (0.2 M Na_2HPO_4 and 0.1 M TCEP·HCl) and methoxyamine·HCl added to attain a concentration of 0.2 M methoxyamine·HCl. The pH of the solution was then adjusted to 4 and the mixture shaken for 18 h at r.t. to afford **17**. The reaction progress was monitored by LC-MS: t_R (**16**) = 11.1 min, $[M + H]^+$ found 1096.3, $[C_{43}H_{61}N_{13}O_{17}S + H]^+$ requires 1096.4; t_R (**17**) = 11.1 min, $[M + H]^+$ found 1084.4, $[C_{43}H_{61}N_{13}O_{17}S + H]^+$ requires 1084.4 (**Figure S6**).

Upon completion of the reaction, the pH was adjusted to 7.5 with aq. NaOH (0.1 M) to effect cyclisation to **11** and monitored by LC-MS (**Figure S7**). Upon reaction completion (*ca* 30 min), the cyclisation mixture was acidified to pH 2 before purification according to **General Method E** to afford a white flaky powder (24.3 mg, 39% yield, 98% purity): RP-HPLC: t_R = 7.5 min; ESI-MS: $[M + H]^+$ found 921.3, $[C_{43}H_{62}N_{12}O_{16}S + H]^+$ requires 921.3, **Figure S8**.

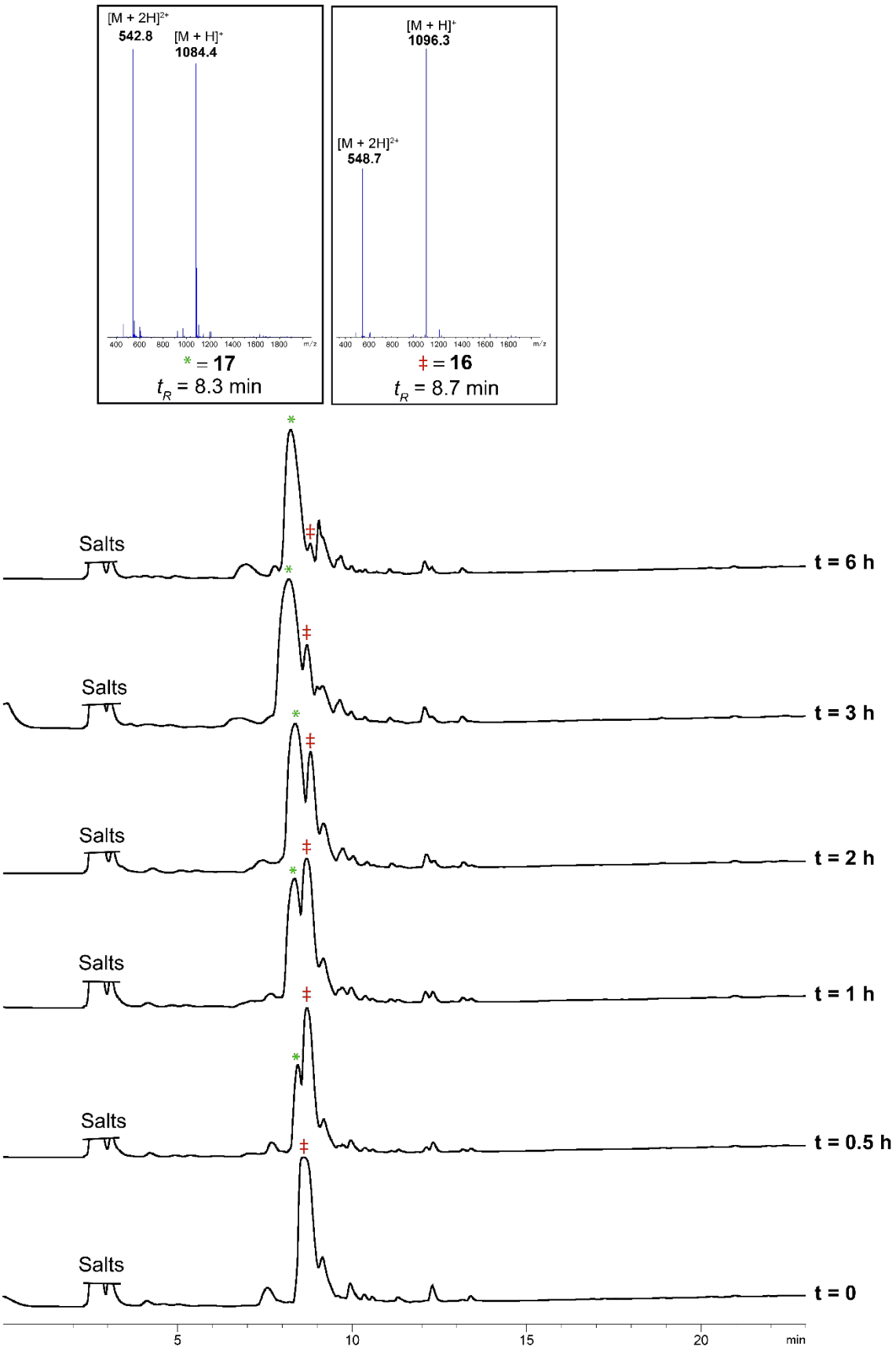


Figure S6. LC-MS time profile of Thz deprotection from t = 0 to t = 6 h. * = unmasked peptide **17**, ‡ = starting material **16**. LC-MS was performed with Agilent Zorbax 300SB-C3 (3.5 µm, 3.0 × 150 mm), linear gradient of 5% B to 65% B over 20 min, *ca.* 3% B per minute at r.t., 0.3 mL/min.

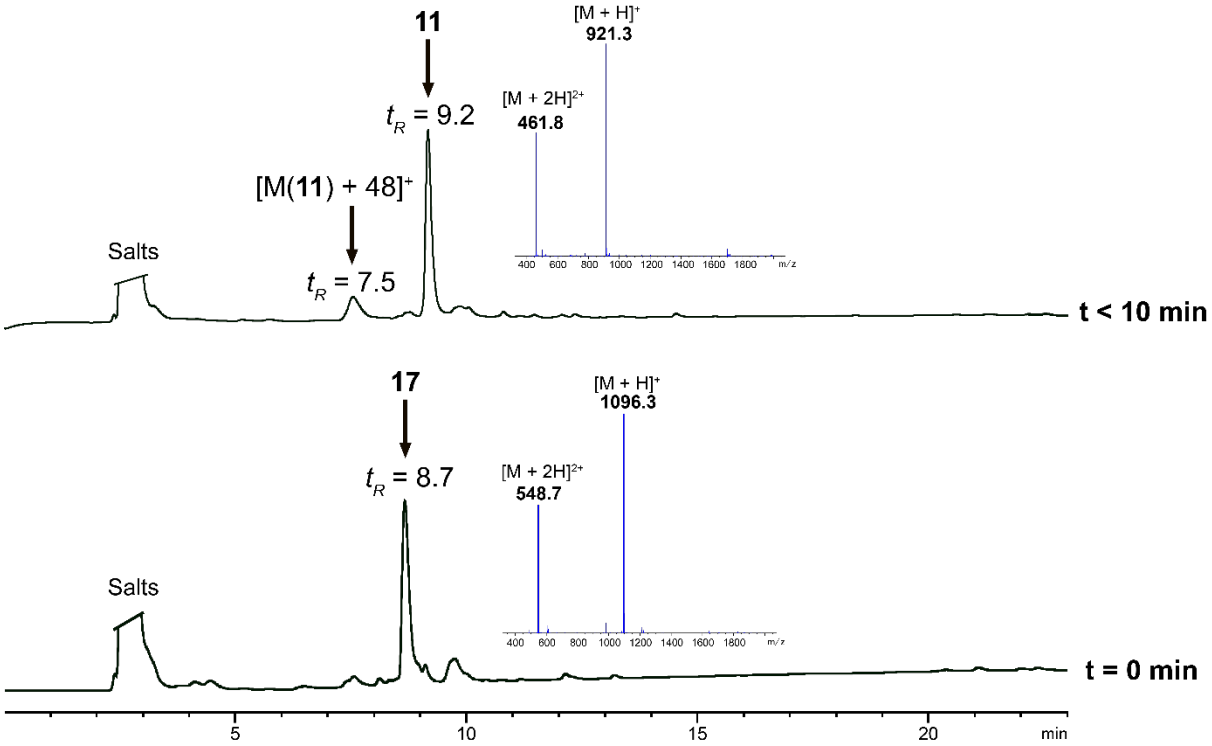


Figure S7. LC-MS time profile of cyclisation of **16** using NCL to yield cyclic peptide **11.** LC-MS was performed with Agilent Zorbax 300SB-C3 (3.5 µm, 3.0 × 150 mm), linear gradient of 5% B to 65% B over 20 min, *ca.* 3% B per minute at r.t., 0.3 mL/min.

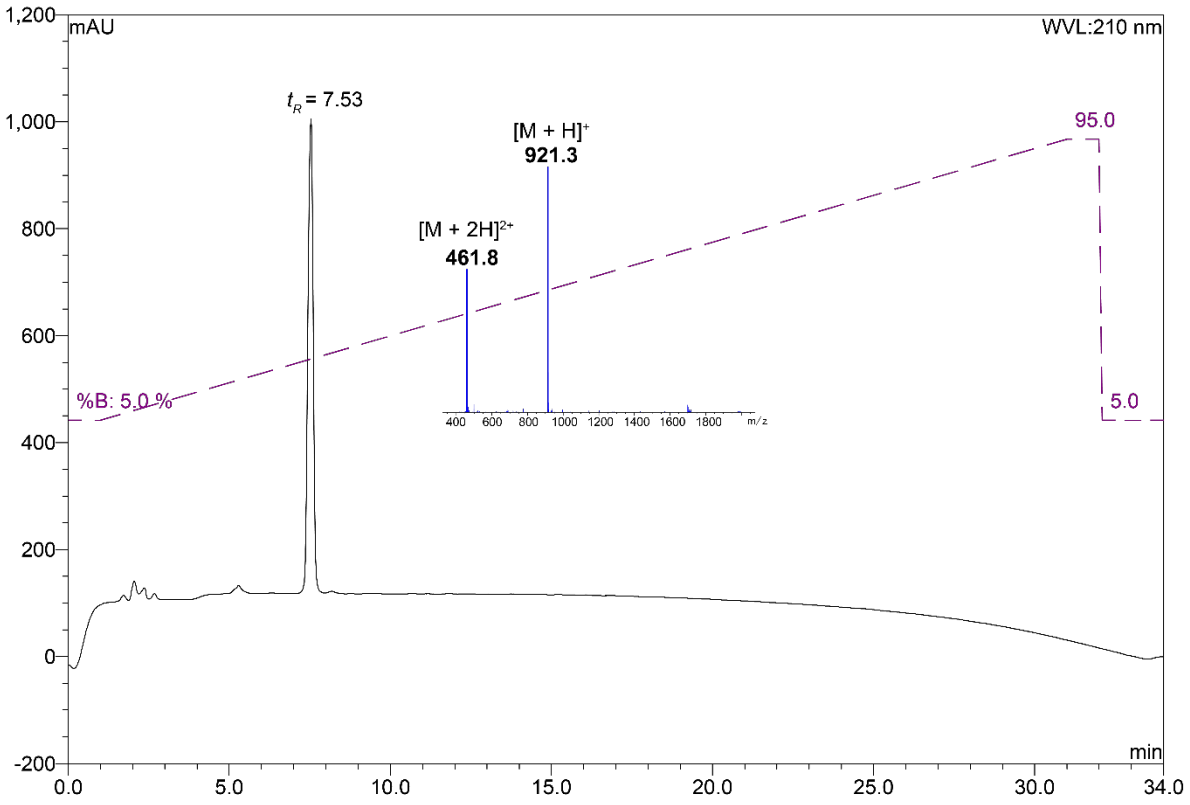
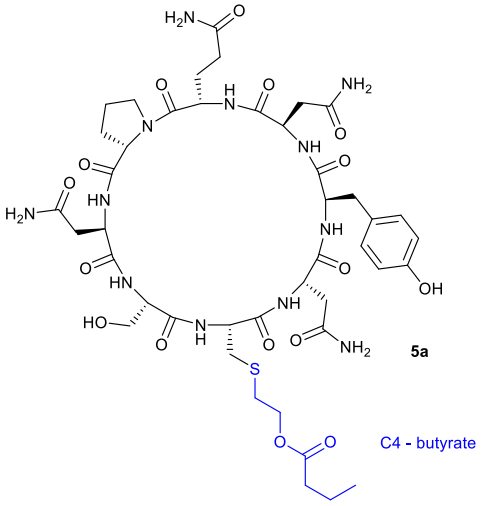


Figure S8. Analytical RP-HPLC trace with inset ESI-MS spectrum of iturin CLipPA mimic **11** (*ca.* 98% as judged by peak area of RP-HPLC at 210 nm); Xterra MS C18 (5 µm, 4.6 × 150 mm), linear gradient of 5% B to 95% B over 30 min, *ca.* 3% B per minute at r.t., 1 mL/min.

S4.3. Generating iturin CLipPA mimics 5

S4.3.1. Synthesis of iturin CLipPA mimic 5a



Purified peptide **11** (11.6 mg, 12.6 µmol), DMPA (3.2 mg, 12.6 µmol, 1 eq.), vinyl butyrate (112 µL, 0.9 mmol, 70 eq.) and TIPS (207 µL, 1.0 mmol, 80 eq.) were dissolved in NMP (1.16 mL, 10 mg/mL final concentration w.r.t. **11**) and degassed with argon for 15 min. Volatile liquids *t*NonSH (189 µL, 1.0 mmol, 80 eq.) and TFA (58 µL, 5% *v/v*) were added under argon. The mixture underwent the CLipPA reaction according to **General Method F**. Purification according to **General Method E** yielded purified iturin CLipPA mimic **5a** (1.2 mg, 9.2% yield, 97% purity) as a white solid; RP-HPLC: t_R = 9.7 min; ESI-MS: $[M + H]^+$ found 1035.4, $[C_{43}H_{62}N_{12}O_{16}S + H]^+$ requires 1035.4, **Figure S9**.

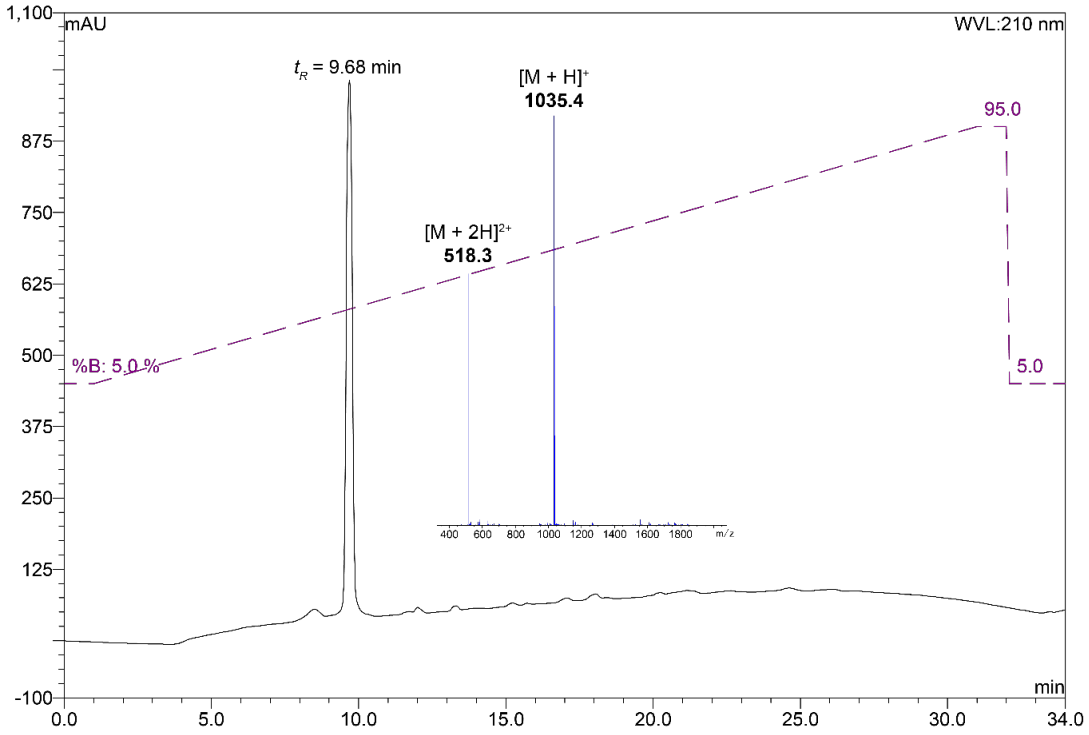
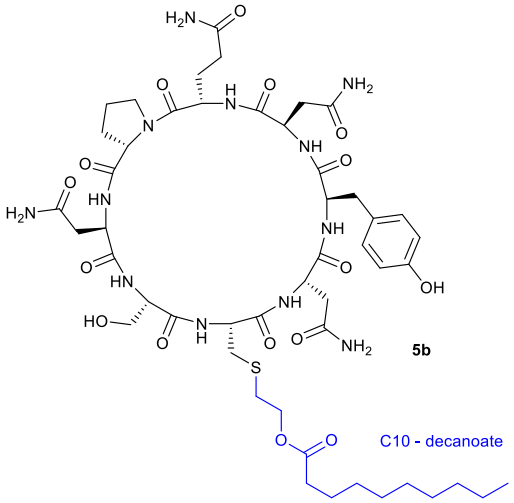


Figure S9. Analytical RP-HPLC trace with inset ESI-MS spectrum of iturin CLipPA mimic **5a** (*ca.* 97% as judged by peak area of RP-HPLC at 210 nm); Xterra MS C18 (5 µm, 4.6 × 150 mm), linear gradient of 5% B to 95% B over 30 min, *ca.* 3% B per minute at r.t., 1 mL/min.

S4.3.2. Synthesis of iturin CLipPA mimic 5b



Purified peptide **11** (13.3 mg, 14.5 µmol), DMPA (3.7 mg, 14.5 µmol, 1 eq.), vinyl decanoate (226 µL, 1.0 mmol, 70 eq.) and TIPS (237 µL, 1.2 mmol, 80 eq.) were dissolved in NMP (1.33 mL, 10 mg/mL final concentration w.r.t. **11**) and degassed with argon for 15 min. Volatile liquids *t*NonSH (217 µL, 1.1 mmol, 80 eq.) and TFA (67 µL, 5% *v/v*) were added under argon. The mixture underwent the CLipPA reaction according to **General Method F**. Purification according to **General Method E** yielded purified iturin CLipPA mimic **5b** (1.5 mg, 9.3% yield, 96% purity) as a white solid; RP-HPLC: t_R = 16.7 min; ESI-MS: $[M + H]^+$ found 1119.6, $[C_{49}H_{74}N_{12}O_{16}S + H]^+$ requires 1119.5, **Figure S10**.

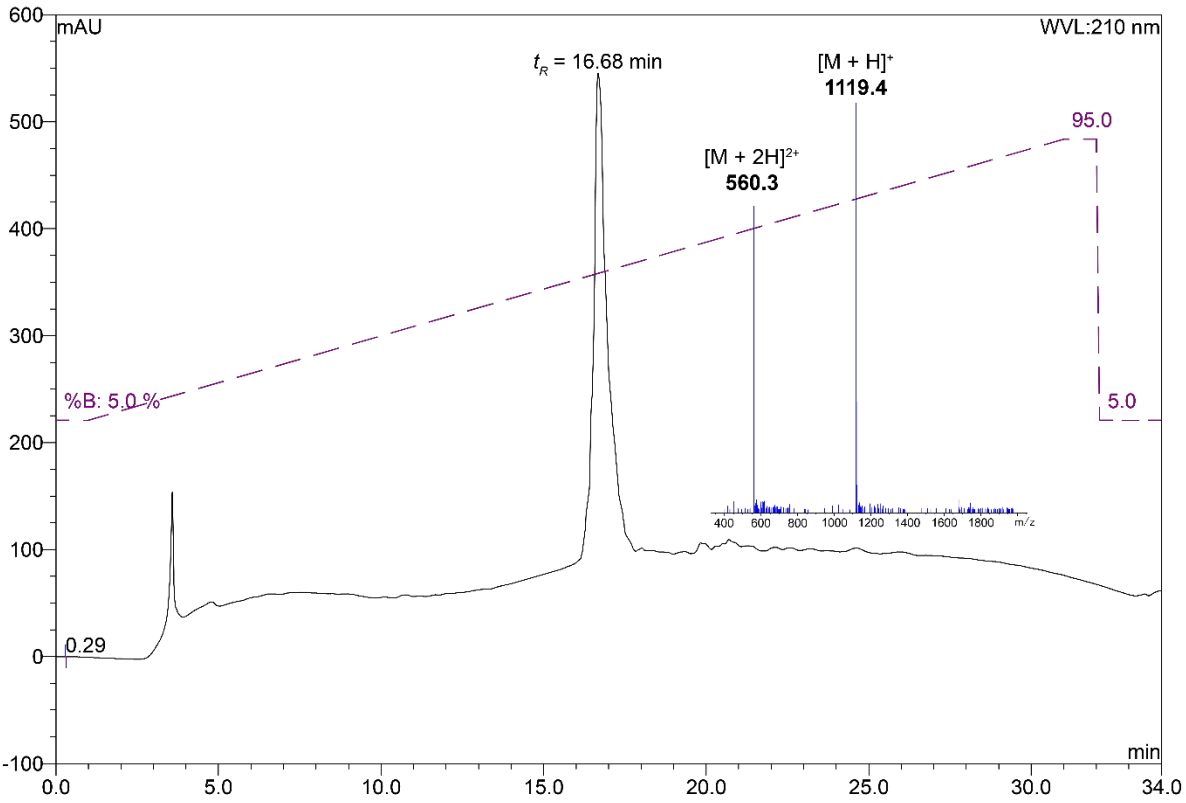
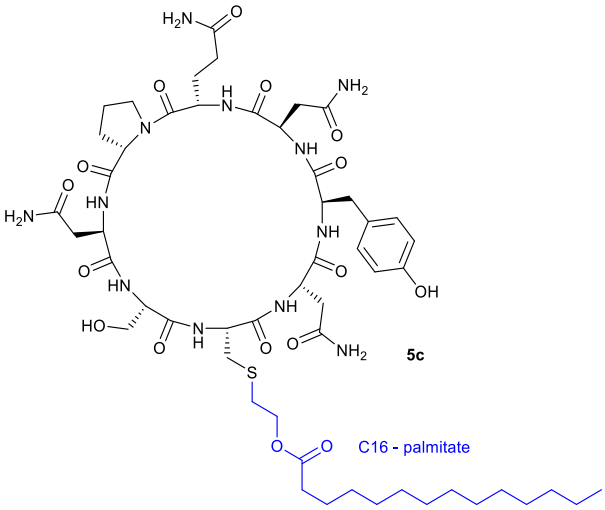


Figure S10. Analytical RP-HPLC trace with inset ESI-MS spectrum of iturin CLipPA mimic **5b** (*ca.* 96% as judged by peak area of RP-HPLC at 210 nm); Xterra MS C18 (5 µm, 4.6 × 150 mm), linear gradient of 5% B to 95% B over 30 min, *ca.* 3% B per minute at r.t., 1 mL/min.

S4.3.3. Synthesis of iturin CLipPA mimic 5c



Purified peptide **11** (14.2 mg, 15.4 µmol), DMPA (4.0 mg, 15.4 µmol, 1 eq.), vinyl palmitate (305 mg, 1.0 mmol, 70 eq.) and TIPS (253 µL, 1.2 mmol, 80 eq.) were dissolved in NMP (1.42 mL, 10 mg/mL final concentration w.r.t. **11**) and degassed with argon for 15 min. Volatile liquids *t*NonSH (231 µL, 1.1 mmol, 80 eq.) and TFA (71 µL, 5% *v/v*) were added under argon. The mixture underwent the CLipPA reaction according to **General Method F**. Purification according to **General Method E** yielded purified iturin CLipPA mimic **5c** (1.1 mg, 5.9% yield, 95% purity) as a white solid; RP-HPLC: t_R = 23.4 min; ESI-MS: $[M + H]^+$ found 1203.6, $[C_{53}H_{82}N_{12}O_{16}S + H]^+$ requires 1203.6, **Figure S11**.

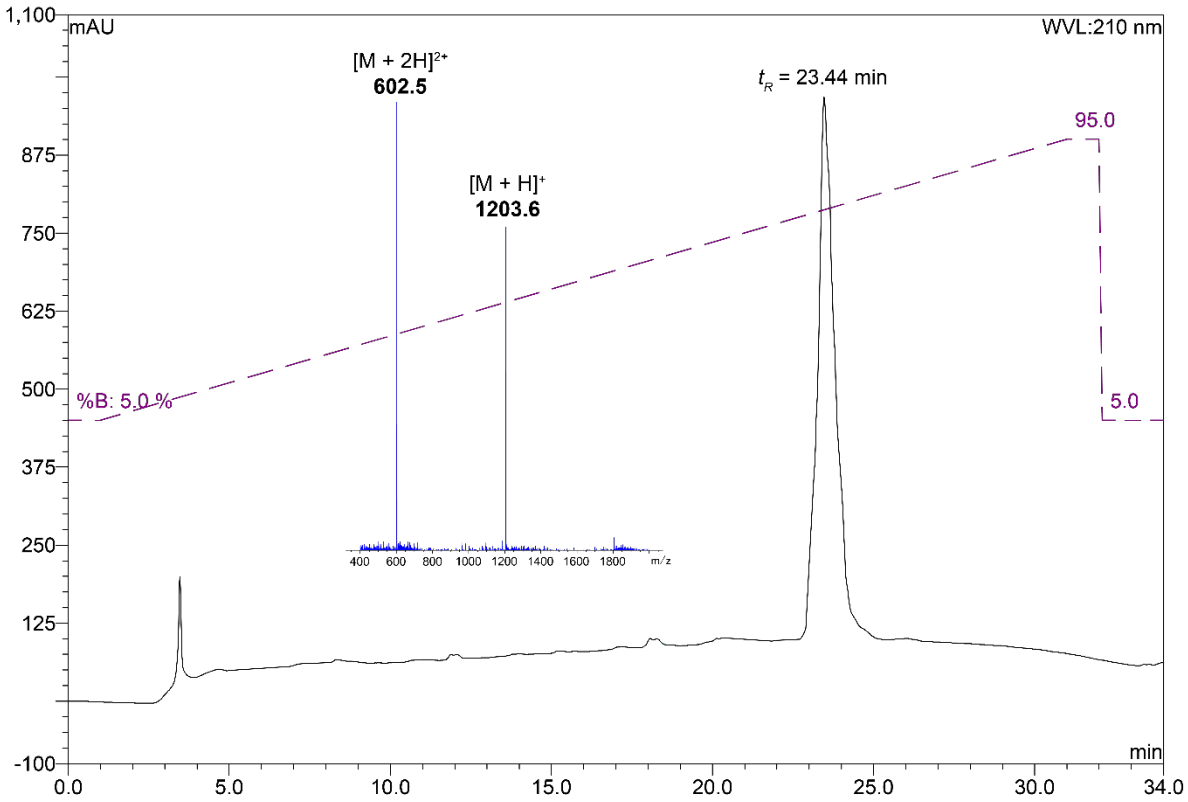
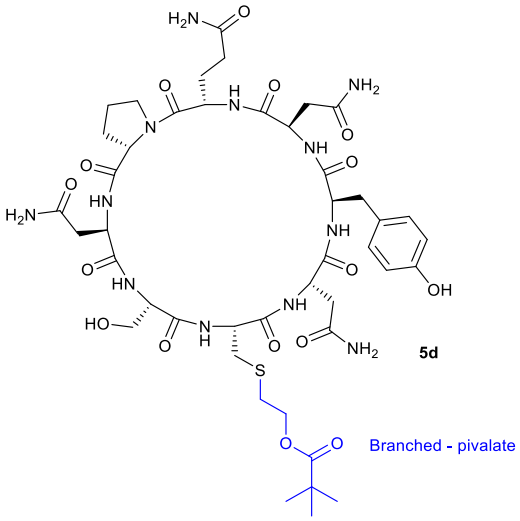


Figure S11. Analytical RP-HPLC trace with inset ESI-MS spectrum of iturin CLipPA mimic **5c** (*ca.* 95% as judged by peak area of RP-HPLC at 210 nm); Xterra MS C18 (5 µm, 4.6 × 150 mm), linear gradient of 5% B to 95% B over 30 min, *ca.* 3% B per minute at r.t., 1 mL/min.

S4.3.4. Synthesis of iturin CLipPA mimic 5d



Purified peptide **11** (14.9 mg, 16.2 µmol), DMPA (4.1 mg, 16.2 µmol, 1 eq.), vinyl pivalate (168 µL, 1.1 mmol, 70 eq.) and TIPS (265 µL, 1.3 mmol, 80 eq.) were dissolved in NMP (1.49 mL, 10 mg/mL final concentration w.r.t. **11**) and degassed with argon for 15 min. Volatile liquids *t*NonSH (243 µL, 1.1 mmol, 80 eq.) and TFA (75 µL, 5% *v/v*) were added under argon. The mixture underwent the CLipPA reaction according to **General Method F**. Purification according to **General Method E** yielded purified iturin CLipPA mimic **5d** (1.8 mg, 10.6% yield, 93% purity) as a white solid; RP-HPLC: t_R = 10.7 min; ESI-MS: $[M + H]^+$ found 1049.4, $[C_{44}H_{64}N_{12}O_{16}S + H]^+$ requires 1049.4, **Figure S12**.

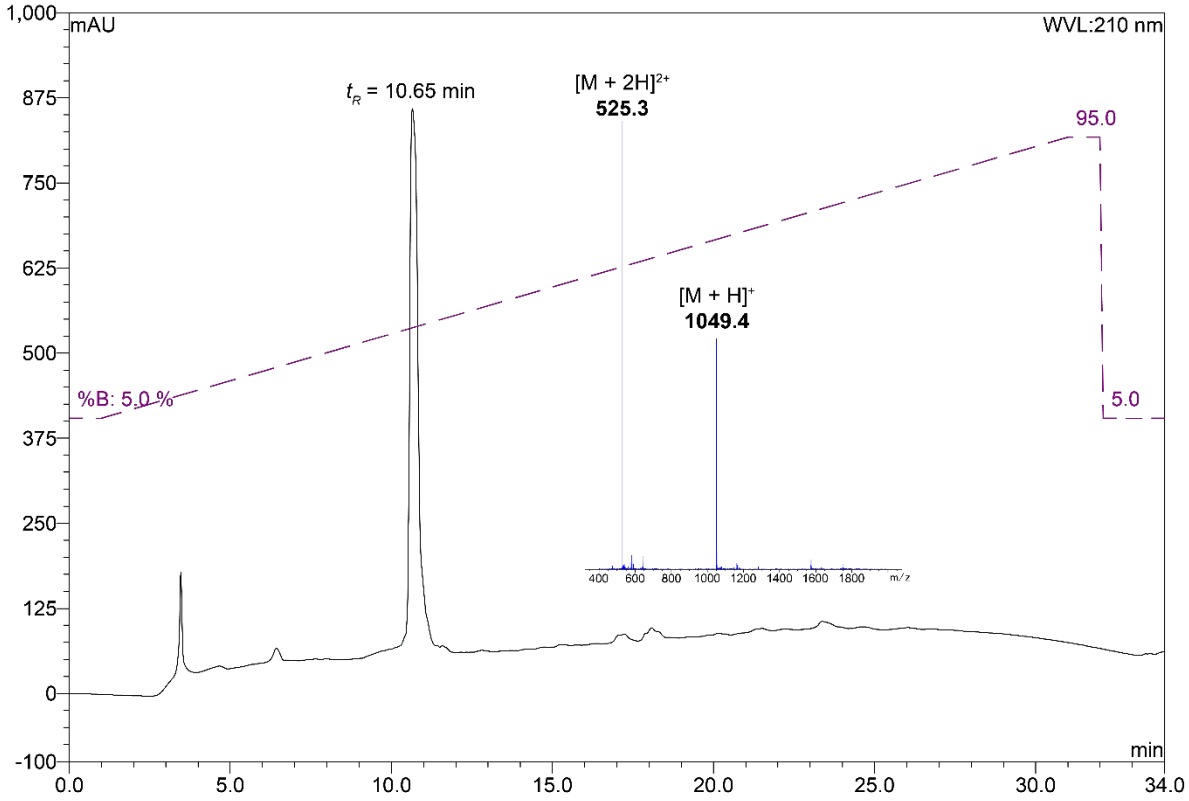
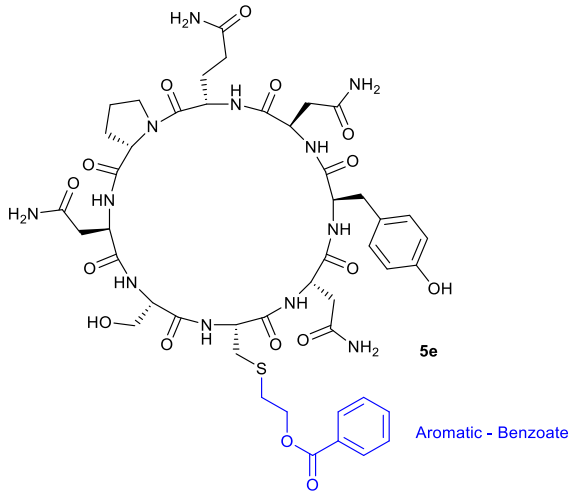


Figure S12. Analytical RP-HPLC trace with inset ESI-MS spectrum of iturin CLipPA mimic **5d** (*ca.* 93% as judged by peak area of RP-HPLC at 210 nm); Xterra MS C18 (5 µm, 4.6 × 150 mm), linear gradient of 5% B to 95% B over 30 min, *ca.* 3% B per minute at r.t.,1 mL/min.

S4.3.5. Synthesis of iturin CLipPA mimic 5e



Purified peptide **11** (15.6 mg, 17.0 µmol), DMPA (4.3 mg, 17.0 µmol, 1 eq.), vinyl benzoate (164 µL, 1.2 mmol, 70 eq.) and TIPS (278 µL, 1.4 mmol, 80 eq.) were dissolved in NMP (1.56 mL, 10 mg/mL final concentration w.r.t. **11**) and degassed with argon for 15 min. Volatile liquids *t*NonSH (254 µL, 1.4 mmol, 80 eq.) and TFA (78 µL, 5% *v/v*) were added under argon. The mixture underwent the CLipPA reaction according to **General Method F**. Purification according to **General Method E** yielded purified iturin CLipPA mimic **5e** (1.5 mg, 8.3% yield, 94% purity) as a white solid; RP-HPLC: t_R = 11.0 min; ESI-MS: $[M + H]^+$ found 1069.3, $[C_{46}H_{60}N_{12}O_{16}S + H]^+$ requires 1069.4, **Figure S13**.

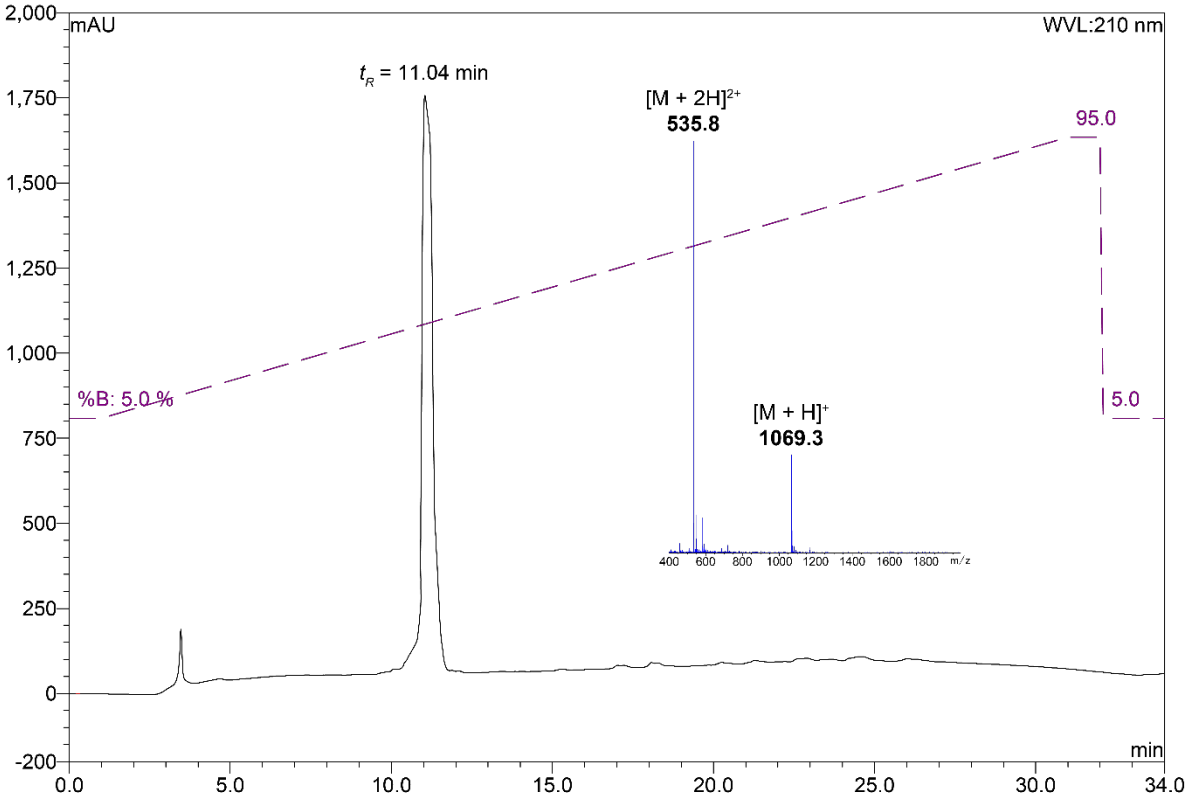
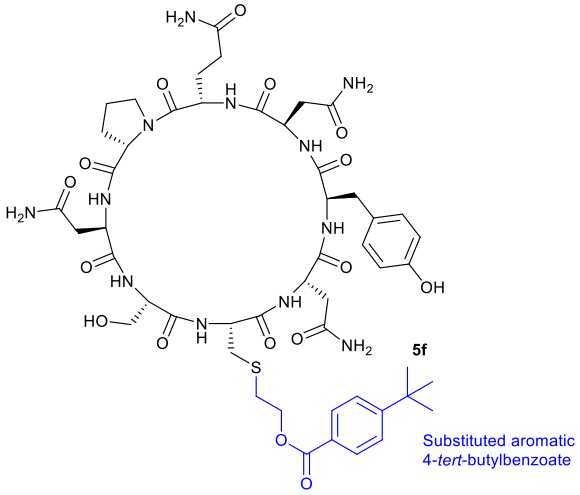


Figure S13. Analytical RP-HPLC trace with inset ESI-MS spectrum of iturin CLipPA mimic **5e** (*ca.* 94% as judged by peak area of RP-HPLC at 210 nm); Xterra MS C18 (5 µm, 4.6 × 150 mm), linear gradient of 5% B to 95% B over 30 min, *ca.* 3% B per minute at r.t., 1 mL/min.

S4.3.6. Synthesis of iturin CLipPA mimic 5f



Purified peptide **11** (16.6 mg, 18.0 µmol), DMPA (4.6 mg, 18.0 µmol, 1 eq.), vinyl 4-*tert*-butylbenzoate (258 µL, 1.3 mmol, 70 eq.) and TIPS (296 µL, 1.4 mmol, 80 eq.) were dissolved in NMP (1.66 mL, 10 mg/mL final concentration w.r.t. **11**) and degassed with argon for 15 min. Volatile liquids *t*NonSH (270 µL, 1.4 mmol, 80 eq.) and TFA (83 µL, 5% *v/v*) were added under argon. The mixture underwent the CLipPA reaction according to **General Method F**. Purification according to **General Method E** yielded purified iturin CLipPA mimic **5f** (0.9 mg, 4.4% yield, 96% purity) as a white solid; RP-HPLC: t_R = 14.7 min; ESI-MS: $[M + H]^+$ found 1125.4, $[C_{50}H_{68}N_{12}O_{16}S + H]^+$ requires 1125.5, **Figure S14**.

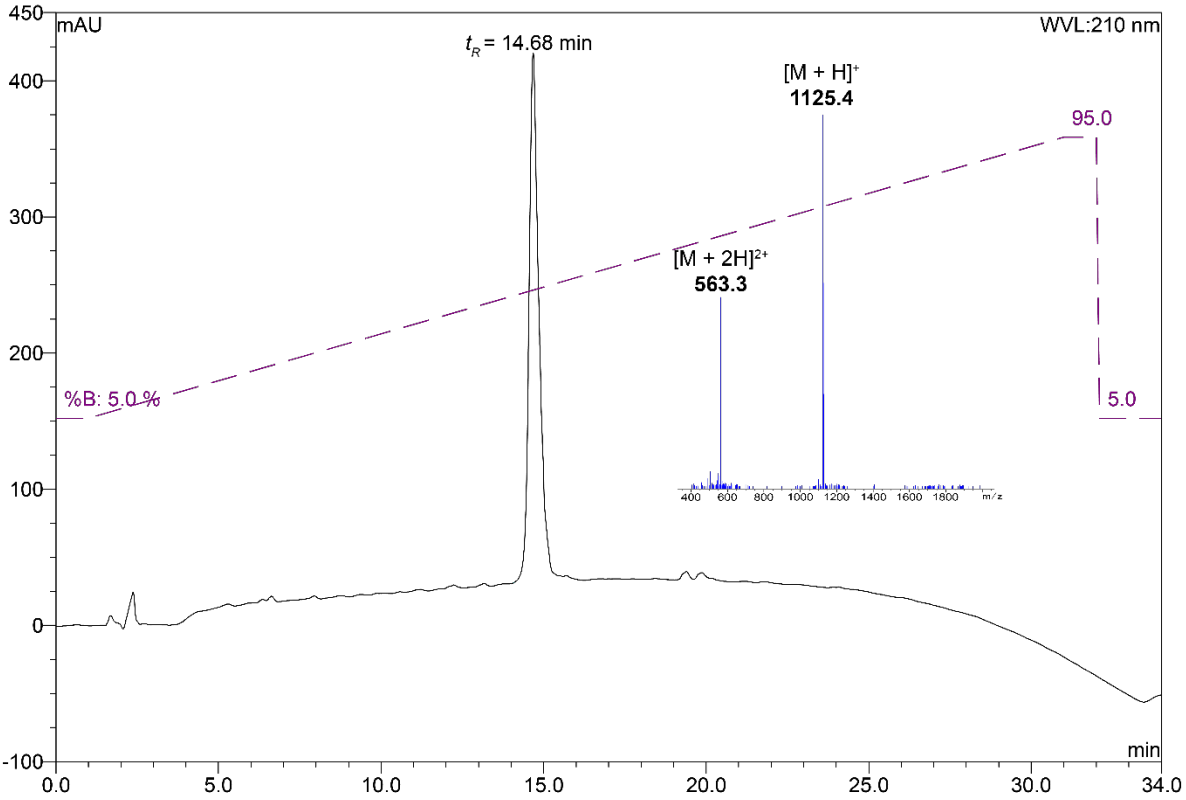


Figure S14. Analytical RP-HPLC trace with inset ESI-MS spectrum of iturin CLipPA mimic **5f** (*ca.* 96% as judged by peak area of RP-HPLC at 210 nm); Xterra MS C18 (5 µm, 4.6 × 150 mm), linear gradient of 5% B to 95% B over 30 min, *ca.* 3% B per minute at r.t., 1 mL/min.

S4.4. Antifungal susceptibility testing

MIC assays were performed with *C. albicans* SC5314 in accordance with the CLSI M27-A2 broth microdilution protocol, using polypropylene 96-well plates.[6] After incubation with a series of two-fold compound dilutions at 37 °C for 48 h, the MIC was determined by visual inspection. Amphotericin B and commercially available iturin A were employed as controls. Compounds were assayed in triplicate and the experiment repeated three times. The MIC was defined as the lowest concentration at which no growth was observed (**Table S1**).

Table S1. MIC Iturin analogues **5a – 5f** in µM

Peptide	*C. albicans* SC5314
5a	> 128
5b	> 128
5c	> 128
5d	> 128
5e	> 128
5f	> 128
Iturin A (commercial)	32
Amphotericin B	2

S5. References

1 V. K. Sarin, S. B. H. Kent, J. P. Tam and R. B. Merrifield, *Anal. Biochem.*, 1981, **117**, 147–157.
2 R. D. Dimarchi, J. P. Tam, S. B. H. Kent and R. B. Merrifield, *Int. J. Pept. Protein Res.*, 1982, **19**, 88–93.
3 P. W. R. Harris, D. J. Lee and M. A. Brimble, *J. Pept. Sci.*, 2012, **18**, 549–555.
4 W. C. Chan and P. D. White, in *Fmoc solid phase peptide synthesis: a practical approach*, eds. W. C. Chan and P. D. White, Oxford University Press, 2000, pp. 41–76.
5 M. W. Pennington, in *Peptide Synthesis Protocols*, eds. M. W. Pennington and B. M. Dunn, Humana Press, Totowa, NJ, 1995, pp. 41–62.
6 Clinical and Laboratory Standards Institute. Reference method for broth dilution antifungal susceptibility testing of yeasts; approved standard – second edition. CLSI document M27-A2, Wayne, PA (2002)