COMMUNICATION

Solid-phase peptide synthesis on disulfide-linker resin followed by reductive release affords pure thiol-functionalized peptides

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Zsolt Bognar,[1,2] Ganesh K. Mothukuri,[1,2] Alexander L. Nielsen,[1] Manuel L. Merz,[1] Peter M. F. Pânzar[1] and Christian Heinis[1,*]

Thiol groups are suitable handles for site-selectively modifying, immobilizing or cyclizing individual peptides or entire peptide libraries. A limiting step in producing the thiol-functionalized peptides is the chromatographic purification, which is particularly laborious and costly if many peptides or even large libraries are to be produced. Herein, we present a strategy in which thiol-functionalized peptides are obtained in > 90% purity and free of reducing agent, without a single chromatographic purification step. In brief, peptides are synthesized on solid support linked via a disulfide bridge, the side-chain protecting groups are eliminated and washed away while the peptides remain on resin, and rather pure peptides are released from the solid support by reductive cleavage of the disulfide linker. Application of a volatile reducing agent, 1,4-butanedithiol (BDT), enabled removal of the agent by evaporation. We demonstrate that the approach is suited for the parallel synthesis of many peptides and that peptides containing a second thiol group can directly be cyclized by bis-electrophilic alkylating reagents for producing libraries of cyclic peptides.

Sulfhydryls, also called thiols, are widely used as functional groups in peptides for labeling, immobilizing or cyclizing individual peptides or libraries of peptides.[1] Several thiol-reactive chemical groups such as maleimides, haloacetamides, vinylsulfoxides, bromomethylbenzenes and pyridyl disulfides are suited to conjugate fluorophores, radionuclides, biotin and other functional molecules via sulfhydryl groups to peptides.[2] Cross-linker reagents containing the same thiol-reactive groups are used to conjugate peptides to surfaces, proteins, DNA strands or other macromolecules. Thiol groups are also used for peptide cyclization, wherein two or three thiol groups are introduced into peptides for reaction with bi- or tri-valent cross-lining agents to generate mono- or bicyclic peptides.[3] Our laboratory has cyclized thousands of cysteine-containing peptides that were identified in phage display selections against diverse protein targets.[4] We have also cyclized large libraries of short thiol-functionalized peptides for generating and screening sub-kilodalton macrocyclic compound libraries.[5]

The thiol groups can easily be introduced into peptides during solid-phase peptide synthesis (SPPS) by incorporating cysteines, cysteine analogs, or by appending non-amino acid thiol-building blocks. However, a major bottleneck in producing peptides is the chromatographic purification, in particular for generating large numbers of peptides. In order to omit peptide purification after SPPS, strategies were developed in which thiol-functionalized peptides are deprotected while still linked to the solid phase, allowing washing away the protecting groups and releasing peptides in a rather pure form.[6–8] Tegge and co-workers synthesized cyclic peptides on a solid support via a disulfide linker and released them by reducing the disulfide bond by dithiothreitol (DTT).[6] Gless and Olsen produced thiol-functionalized cyclic peptides applying Dawson's 3-amino-4-(methylamino)benzoic acid (MeDbz) linker[9] which yielded unprotected thioester peptides that were cyclized by native chemical ligation.[7] Our laboratory has recently developed a strategy for efficiently accessing disulfide-cyclized peptides by synthesizing peptides via a disulfide linker on solid phase and releasing them via a cyclative disulfide exchange reaction.[8]

In peptides produced with all the described strategies, the thiol groups tend to oxidize during the production or storage, or are fully oxidized such as in the cyclative release approach, and thus require to be treated with reducing agents prior to conjugation reactions. The reducing agents applied for breaking the disulfide bridges, however, require to be removed afterwards as they would interfere with the chemical reactions, and this involves a cumbersome purification step. Phosphine-based reducing agents such as tris-(2-carboxyethyl)phosphine (TCEP) were reported to have a lower reactivity towards thiol-reactive reagents and were used successfully in many bioconjugation applications without removal,[10] but they interfere with thiol-conjugation reactions too[11,12] and proved to not be a viable option for cyclizing thiol-containing peptides in peptide drug development applications of our laboratory.

[a.1] *Institute of Chemical Sciences and Engineering, School of Basic Sciences, Ecole Polytechnique Fédérale de Lausanne (EPFL), CH-1015 Lausanne, Switzerland.*
[b.2] *These authors contributed equally to this work*
[c.] **Correspondence should be addressed to C.H. E-mail: christian.heinis@epfl.ch*
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Herein, we proposed to synthesize peptides on a disulfide linker resin and to release them using a volatile reducing agent that could be removed by evaporation, and thus would not need a purification step prior to chemical modification of the thiol group(s). We show that this strategy allowed for efficient production of thiol-functionalized peptides and enabled the cyclization of the peptides by bis-electrophilic reagents without a single chromatographic purification step.

We first aimed at testing if peptides synthesized via a disulfide linker on a solid support can be released by a volatile reducing agent and if the latter one can be quantitatively removed from the eluted peptide by evaporation (Figure 1a). Towards this end, we synthesized the four peptides Ala-Trp-Mea, Tyr-Ala-Mea, Trp-Ala-Mea and Ala-Tyr-Mea that were linked to thiol-functionalized resin via the C-terminal 2-mercapto-ethylamine (Mea) group (Figure 1b and Supplementary Figure 1a). The peptides contained a tryptophan or tyrosine residue to allow precise determination of the amount of released peptide by absorbance measurement at 280 nm. We synthesized the peptides on polystyrene (PS) resin that is commonly used for peptide synthesis. We introduced the Mea building block in a disulfide exchange reaction using 2-(2-pyridyldithio)ethylamine hydrochloride and thiol-PS resin, as described before.[8] All peptides were synthesized in wells of a 96-well plate on a 5 μmol scale, in order to test the conditions at which we later planned synthesizing peptide libraries at high-throughput.

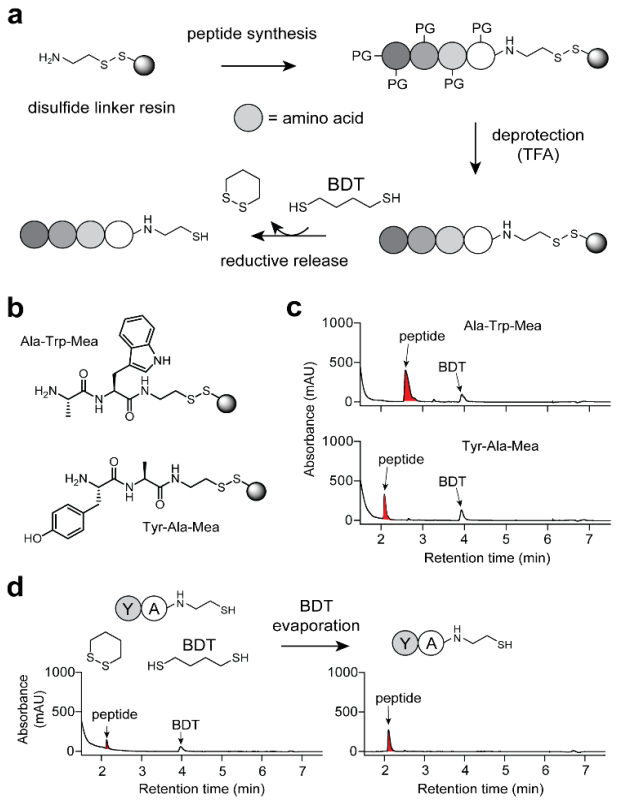

Figure 1. Strategy for the reductive release of peptides synthesized on solid phase via a disulfide bridge. (a) Overview of the synthetic steps. (b) Chemical structures of peptides used to test the reductive release of peptides from solid phase. Mea = mercaptoethylamine (c) HPLC chromatograms of peptides released by reduction with BDT. (d) Removal of reducing agent by centrifugal vacuum evaporation.

We tested the release of disulfide-immobilized peptide from PS resin by incubating the beads overnight with 200 μl DMF containing 20 equivalents of the volatile reducing agent β-mercaptoethanol (β-Me; 0.5 M). LC-MS analysis of the peptides showed efficient release but also that up to 40% of the product occurred disulfide-linked to β-Me (Supplementary Figure 1b). We reasoned that the extent of adduct could potentially be reduced by using a larger molar excess of β-Me and/or by repeating the reduction, but as this would require additional working steps, we looked for a solution based on a different reducing agent. The reducing agent DTT reacts via a disulfide exchange reaction as β-Me but does not stop at the mixed-disulfide species because the second thiol of DTT has a high propensity to close, forming oxidized DTT (as a 6-membered ring) and leaving behind a reduced disulfide bond. Incubation of the resin carrying either peptide Ala-Trp-Mea or Tyr-Ala-Mea with four equivalents of DTT efficiently released the peptides without forming peptide-reducing agent adducts. However, we found that DTT could not be removed by vacuum evaporation in a standard rotary vacuum concentrator due to its rather high boiling point of 365 °C (Supplementary Figure 2a).

A reducing agent that eliminates itself in the same way as DTT by forming a 6-membered ring in the oxidized form, but that evaporates at 195 °C, and thus has a lower boiling point, is 1,4-butanedithiol (BDT).[13] For BDT, a boiling point of 106 °C was reported under vacuum at 40 mbar,[14] and we thus expected that it could be removed at a pressure of 0.1 mbar without the need for highly elevated temperatures, using centrifugal vacuum evaporation. We incubated 5 μmol of the resin-linked peptides with 200 μl DMF containing four equivalents BDT (100 mM). The peptides were efficiently released by BDT as analyzed by LC-MS (Figure 1c and Supplementary Figure 2b). For all the four peptides, a major peak corresponding to the desired product, was observed. The yields of the desired products were 3.4, 1.3, 4.2 and 4.3 μmol, respectively, which corresponded to 68, 25, 84 and 86% yield (based on 5 μmol resin loading). Pleasingly, the excess of BDT and oxidized BDT could efficiently be removed by centrifugal evaporation under vacuum on a SpeedVac (Figure 1d).

We next aimed at applying the reductive release strategy for synthesizing short dithiol peptides, which can be efficiently cyclized by bis-electrophile reagents such as **1**, and which are of great interest for the generation of small, membrane-permeable macrocycles (Figure 2a). In previous attempts to produce short dithiol peptides using a cyclative disulfide release strategy (Figure 2b),[8] we had difficulties to obtain peptides in good yields, likely due to the short length of the peptides which sterically hinders the intramolecular disulfide exchange reaction. With the new approach releasing the peptides by disulfide bond reduction, we expected an efficient release as no cyclization was required. We synthesized a panel of eight short dithiol peptides of the format Mpa-Xaa-Mea (Mpa = 3-mercaptopropionic acid), wherein the Xaa amino acids were Trp, Tyr, Ser, His, Phe, Arg, Asp and Ala (Figure 2c). Treatment of the resins with BDT indeed lead to efficient release of all peptides (Figure 2d, left panels, and Supplementary Figure 3). The main products were the desired dithiol peptides. Side

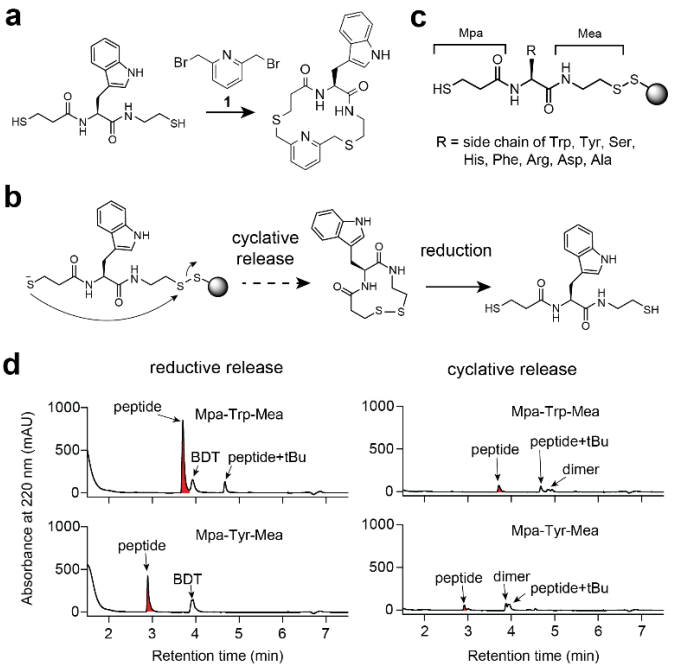

Figure 2. Synthesis of short dithiol peptides for the generation of macrocyclic compound libraries. (a) Cyclization reaction illustrated with the dithiol peptide Mpa-Trp-Mea and the bis-electrophile reagent 2,6-bis(bromomethyl)pyridine (**1**). (b) Recently developed "cyclative disulfide release" reaction.[8] Short peptides such as those containing only one amino acid between the two flanking thiol-containing structures (three building blocks) are not efficiently released due to conformational constraints (indicated by dashed line of arrow). (c) Structure of short dithiol peptides. (d) Comparison of yields obtained by reductive release (left) and cyclative release (right). The peaks of the desired peptides are highlighted in red. HPLC chromatograms of more dithiol peptides are shown in Supplementary Figure 3.

products were found in only small quantities and were dithiol peptides that carried tert-butyl groups. For comparison, we applied conditions to release the peptides via the cyclative release approach (Figure 2b), which yielded the short peptides in around 10 to 100-fold smaller quantities (Figure 2d, right panels, and Supplementary Figure 3) and showed the advantage of reductively releasing the short peptides. As a positive control for the cyclative release method, we applied longer peptides such as Mpa-Lys-Trp-Gly-βAla-Mea which were satisfactorily released via the cyclative release mechanism, most likely due to the smaller conformational constraints (Supplementary Figure 4). The yields of the short peptides Mpa-Trp-Mea and Mpa-Tyr-Mea released by reduction with BDT were 3.6 and 4.3 μmol, respectively, which corresponded to 71% and 85% yield. Taken together, the reductive release strategy with BDT allowed accessing short dithiol peptides that could not be obtained in sufficient yields with the previously applied cyclative release approach.

We next tested if the reducing agent BDT can be removed from the dithiol peptides by centrifugation under vacuum, which was required for the subsequent cyclization of the peptides by bis-electrophile reagents. Peptides synthesized at a scale of 5 μmol and released in 200 μl DMF containing 100 mM BDT and 100 mM TEA were centrifuged at 0.1 mbar, 30 °C and at 400 × *g* in 96-well plates. The solvent was efficiently removed in less than one hour, even if all wells of the microwell plate were filled. LC-MS analysis of the peptides showed that all BDT was removed. However, some peptides had up to 10% of back-oxidized product. We speculated that the oxidation was enabled by the alkaline pH, and we thus added two equivalents of TFA relative to TEA to each well prior to the centrifugal vacuum evaporation. With this acidification procedure, the fraction of oxidized peptide could be reduced efficiently (Supplementary Figure 5).

We tested if the short dithiol peptides Mpa-Trp-Mea and Mpa-Tyr-Mea could be cyclized by a panel of ten bis-electrophile reagents (reagents **1** to **10**; Figure 2a, 3a and Supplementary Figure 6). The cyclization of peptides via two or three cysteines by electrophilic linker reagents is highly efficient and clean if performed at dilute concentrations, with the peptides around 1 mM (or lower) and the cyclization reagents applied at a small excess.[15,16] We dissolved the peptides in 1 ml MeCN:water 1:1, added 3 ml of NH_4HCO_3 buffer (100 mM, pH 8.0) containing 10% acetonitrile, and immediately added 1 ml bis-electrophile reagents in acetonitrile (10 mM). The final concentrations of peptide and cyclization reagent were around 1 mM and 2 mM, respectively. The HPLC chromatograms of the cyclization reactions with the peptide Mpa-Trp-Mea are shown in Figure 3b and Supplementary Figure 6, and those with the peptide Mpa-Tyr-Mea in the Supplementary Figure 7. In most of the

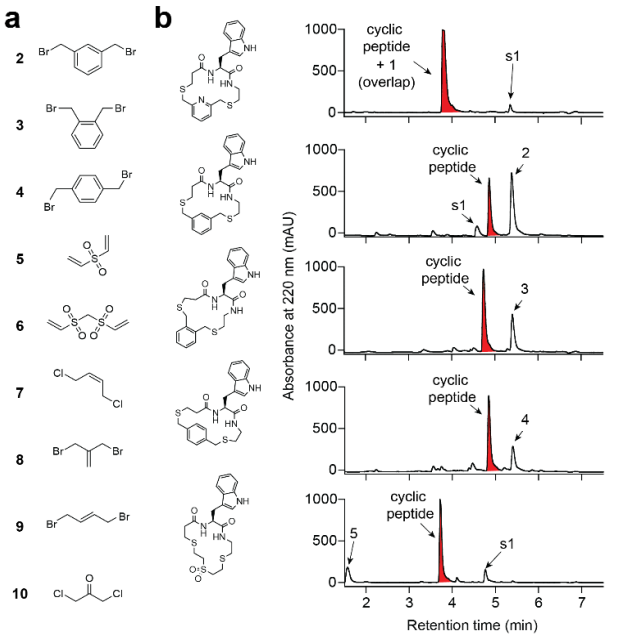

Figure 3. Cyclization of dithiol peptides by bis-electrophile reagents. (a) Bis-electrophile reagents **2**–**10**. (b) HPLC chromatographic analysis of Mea-Trp-Mpa cyclized by reagents **2**–**5**. The desired cyclic products are highlighted in red. s1 = side-product tert-butyl-peptide-linker (structure shown in Supplementary Figure 8). Data for cyclizations with reagents **6**–**10** are shown in Supplementary Figure 6.

reactions, the dithiol peptides were cyclized nearly quantitatively with yields higher than 90%. The small quantity of side product was mainly peptide that reacted with only one thiol group because one of them was modified with tert-butyl derived from side chain protecting groups (Supplementary Figure 8). Addition of six equivalents of β-Me (relative to peptide) allowed efficient quenching of the excess of bis-electrophile reagents (Supplementary Figure 9).

A second goal of this study was to assess if slightly longer peptides could be synthesized on a disulfide linker solid phase. In our previous work using the above described cyclative release strategy, we had tested the synthesis of rather short peptides having 2–3 amino acids, and it was not clear if the synthesis of longer peptides would be feasible, as the disulfide linker is exposed many more times to piperidine and could potentially break. In order to test the synthesis of longer peptides, we produced alanine-scan mutants of a cyclic peptide ligand of the cancer target KRAS, that was previously identified by phage display[17] and that is of interest to our laboratory (Figure 4a). We synthesized the undecamer CPLYISYDPVC with Mpa and Mea in place of the N- and C-terminal cysteines. Despite the ten Fmoc deprotection steps that exposed the disulfide linker resin to the base piperidine, the peptides were obtained in good yields ranging from 28% to 59% based on resin loading, indicating that the disulfide bridges resist to a large extent the repetitive treatment with the base (Figure 4b and Supplementary Figure 10). Centrifugal evaporation under vacuum again efficiently removed BDT and allowed efficient peptide cyclization with a bis-electrophilic cyclization reagent (**10**) (Figure 4c and 4d). The example with several different peptides from the alanine-scan showed that peptides containing around 10 amino acids, and likely even longer ones, can be synthesized efficiently with the new method.

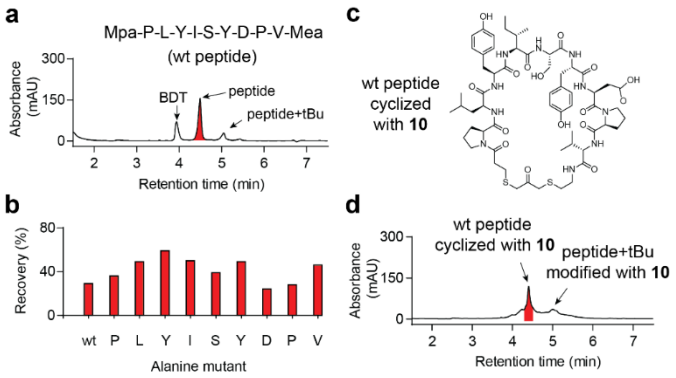

Figure 4. Synthesis of longer peptides, tested with sequences based on the phage display-selected KRAS-specific peptide KRpep-2a. (a) HPLC chromatogram of linear peptide containing the core sequence of KRpep-2a (wt peptide) (b) Yields of alanine mutants of KRpep-2a indicated as % recovery relative to the resin loading. HPLC chromatograms are shown in Supplementary Figure 10. (c) Chemical structure of the wt peptide cyclized with linker **10**. (d) HPLC chromatogram of the wt peptide cyclized with linker **10**.

In conclusion, we were able to establish an SPPS and elution strategy that delivers thiol-functionalized peptides in high yields and purities, all without the need of a chromatographic purification step. Importantly, the strategy involves an easy and efficient step to remove the reducing agent needed for peptide release, allowing to directly conjugate or cyclize the thiol-functionalized peptides with electrophilic reagents. We show that the strategy can be applied for the synthesis of peptides having at least ten amino acids. Given the wide use of thiol-functionalized peptides in research, the new method may be broadly applied for more efficiently accessing peptides needed in diverse applications, ranging from peptide production for alanine scans, peptide array generation for linear and non-linear epitope scanning (similar to SPOT synthesis), synthesis of the numerous peptides identified by in vitro evolution using phage- or mRNA display selections, or the generation of cyclic peptide macrocycle libraries. The herein presented volatile reducing agent BDT, that we report can be efficiently removed by centrifugal vacuum evaporation, might be broadly applied to other molecules than peptides, such as for breaking disulfide bridges of proteins or reducing thiol-functionalized DNA strands or carbohydrates.

Acknowledgements

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Conflicts of interest

Z.B., G.K.M., A.L.N., M.L.M. and C.H. are inventors of a patent application covering the new method. C.H. is a co-founder of Orbis Medicines. P.M.F.P. declares no potential conflict of interest.

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SUPPORTING INFORMATION

Solid-phase peptide synthesis on disulfide-linker resin followed by reductive release affords pure thiol-functionalized peptides

Zsolt Bognar,[1,2] Ganesh K. Mothukuri,[1,2] Alexander L. Nielsen,[1] Manuel L. Merz,[1] Peter M. F. Pânzar[1] and Christian Heinis[1,*]

[1]Institute of Chemical Sciences and Engineering, School of Basic Sciences, Ecole Polytechnique Fédérale de Lausanne (EPFL), CH-1015 Lausanne, Switzerland.

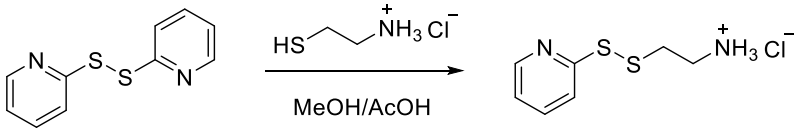
[2]These authors contributed equally to this work

*Correspondence should be addressed to C.H. E-mail: christian.heinis@epfl.ch

Contents:

SUPPLEMENTARY MATERIALS AND METHODS

Synthesis of 2-(2-pyridyldithio)ethylamine hydrochloride



To a stirring solution of 2,2'-dipyridyldisulfide (4.41 g, 20 mmol) in MeOH (16 ml with 2% [v/v] AcOH) was added cysteamine hydrochloride (1.14 g, 10 mmol) dissolved in MeOH (10 ml, with 2% [v/v] AcOH dropwise over 15 min. The reaction mixture was stirred overnight at room temperature (RT) and concentrated under reduced pressure to afford a yellow oil. The residue was dissolved in MeOH (16 ml), distributed to eight 50-ml falcon tubes, and precipitated by addition of ice-cold diethyl ether (48 ml to each tube). The tubes were incubated at -20 °C for 30 min and centrifuged at 3400 × *g* (4000 rpm on an explosion proof Sigma centrifuge) at 4 °C for 30 min. The product was obtained as a colorless solid after repeating the precipitation 8 times (yield = 90%).

Preparation of cysteamine-polystyrene resin

The following procedure describes the preparation of polystyrene resin carrying around 2 mmol cysteamine immobilized via a disulfide linker, which is sufficient for the synthesis of 4 × 96 peptides at a 5 μmol scale. Into each one of four 20 ml plastic syringes was added 563 mg resin (Rapp Polymere Polystyrene A SH resin, 200-400 mesh, 0.95 mmol/gram loading). The resin was washed with DCM (15 ml), then swelled in MeOH/DCM (7:3; 15 ml) for 20 min. Pyridyl-cysteamine disulfide (2.10 grams, 9.42 mmol, 4.4 equiv.) was dissolved in MeOH (23 ml) and then DCM (53 ml). Then *N,N*-diisopropylethylamine (DIPEA; 410 μl) was added. A volume of 19 ml of this solution was pulled into each syringe and the syringes were then shaken at RT for 3 hours. The pyridyl-cysteamine solution was discarded and the resin was washed with MeOH/DCM (7:3; 2 × 20 ml), then with DMF (2 ×. 20 ml). The resin was combined into a single syringe as a suspension in DMF, washed with a solution of 1.2 M DIPEA in DMF (11.8 ml) for 5 min to ensure that all amines were neutralized. This solution was discarded, and the resin was washed with DMF (2 × 20 ml), then with DCM (4 × 20 ml), then kept under vacuum overnight to yield a free-flowing powder.

Fmoc peptide synthesis in 96-well plates

Peptides were synthesized at a 5 μmol scale in 96-well peptide synthesis filter plates (Orochem, cat. # OF1100) using an automated peptide synthesizer (Intavis MultiPep RSi). Cysteamine-PS resin (around 5 mg, 0.95 mmol/g, 5 μmol scale) was distributed as powder to each well of the plate. The resin was washed

with DMF (3 × 225 μl). In this and all the following washing steps, the resin was incubated for 1 min. The following reagents were transferred to tubes in the indicated order, mixed, incubated for 1 min, transferred to the resin in the microwell plate, and incubated for 45 min without shaking. Reagents: 50 μl HATU (500 mM in DMF, 5 equiv.), 5 μl *N*-methylpyrrolidone (NMP), 12.5 μl of *N*-methylmorpholine (NMM in DMF, 4 M, 10 equiv.) and 53 μl of amino acid (500 mM in DMF, 5.3 equiv.). The final volume of the coupling reaction was 120.5 μl and the final concentrations of reagents were 208 mM HATU, 415 mM NMM and 220 mM amino acid. Coupling was performed twice. The resin was washed with DMF (1 × 225 μl). Unreacted amino groups were capped by incubation with 5% acetic anhydride and 6% 2,6-lutidine in DMF (100 μl) without shaking for 5 min. The resin was washed with DMF (8 × 225 μl). Fmoc groups were removed by incubation twice DMF (120 μl) containing 20% (v/v) piperidine without shaking for 5 min each. For the synthesis of longer peptide sequences, the incubation time was reduced from 5 min to 2 min, in order to reduce exposure to the base. The resin was washed with DMF (8 × 225 μl). At the end of the peptide synthesis, the resin was washed with DCM (2 × 200 μl).

Peptide side chain deprotection in 96-well plates

For removing protecting groups from amino acid side chains as well as from Mpa, the bottom of the 96-well synthesis plate was sealed by pressing the plate onto a soft 6 mm thick ethylene-vinyl acetate pad, and the resin in each well was incubated with a solution of TFA:TIPS:H_2O (95:2.5:2.5 [v/v/v], around 300 μl). The plates were covered with an adhesive sealing film (iST scientific, QuickSeal Micro, cat. # IST-125-080-LS), then weighed down by placing a weight (1 kg) on top to prevent leakage. After 1 h incubation, the synthesis plate was placed onto a 2 ml deep-well plate, and the TFA mixture was allowed to drain. The synthesis plate was again sealed and the deprotection procedure was repeated. The wells were washed with DCM (3 × 500 μl; added with syringe) that was run through the wells by gravity flow. The resin was dried by placing the synthesis plate into a vacuum manifold for 5 min.

Reductive peptide release by β-Me, DTT or BDT

For releasing the peptides from the resin, the bottom of the 96-well synthesis plate was sealed by pressing the plate onto a soft 6 mm thick ethylene-vinyl acetate pad, and the resin in each well was incubated with a solution of 200 μl DMF containing 500 mM of β-Me, or 100 mM DTT, or 100 mM BDT, and 100 mM TEA for 4 h at RT. After this time, the samples were collected in a 96-deep well plate by centrifugation at 250 × *g* (1100 rpm on a Thermo Scientific Heraeus Multifuge 3L-R centrifuge with a Sorvall 75006445 Rotor, radius = 19.2 cm rotor) for 2 min at RT.

Cyclative release of peptides

The 96-well synthesis plate was sealed as described above and the peptides were released from the resin by incubation with a solution of 200 μl DMSO containing 250 mM TEA (10 equiv.) overnight at RT. After this time, the samples were collected in a 96-deep well plate by centrifugation at 250 × *g* (1100 rpm on a Thermo Scientific Heraeus Multifuge 3L-R centrifuge with a Sorvall 75006445 Rotor, radius = 19.2 cm rotor) for 2 min at RT.

LC-MS analysis of peptides after solid phase release or cyclization

For peptides released from solid phase (concentration up to 25 mM in DMF or DMSO), 1 μl of peptide was diluted in 60 μl of milliQ H_2O containing 0.05% formic acid. For peptides from cyclization reactions (concentration around 1 mM), 10 μl of the reaction mixture was mixed with 10 μl of milliQ H_2O containing 0.05% formic acid. Samples (10 μl injection) were analyzed on a Shimadzu 2020 single quadrupole LC-MS system using a reverse phase C18 column (Phenomenex Kinetex®, 2.6 μm, 100 Å, 50 × 2.1 mm) and a linear gradient of solvent B (MeCN, 0.05% formic acid) over solvent A (H_2O, 0.05% formic acid) from 0 to 60% in 5 min at a flowrate of 1 ml/min. Absorbance was recorded at 220 nm and masses were analyzed in the positive mode.

Centrifugal vacuum evaporation of reducing agent and solvent

The following example describes a peptide that had a concentration of 20 mM after reductive release. Of the 200 μl peptide released from the solid phase by reduction (in DMF containing 100 mM BDT and 100 mM TEA), 5 μl (0.1 μmol) were transferred to a well of a V-bottom 96-well plate (Ratiolab, 6018321, PP, unsterile). A volume of 7 μl of 1% TFA in water (v/v) was added to each well to reach 2 equiv. of TFA over TEA. This sample was subjected to centrifugal vacuum evaporation using Christ RVC 2-33 CDplus IR instrument to remove the solvent (DMF) and reducing agent (BDT). Samples were centrifuged at 0.1 mbar, 30 °C and at 400 × *g* (1750 rpm in a Christ 124700 rotor with 124708 plate holder inserts, radius = 10.5 cm). The quantity of peptide expected was so low that it could not be expected to be seen by eye, which was the case.

Cyclization of peptides

The reduced and dried peptide (0.1 μmol) was dissolved in 20 μl of 50% acetonitrile, 50%H_2O to reach a concentration of 5 mM. To this solution 60 μl reaction buffer (100 mM ammonium bicarbonate, pH 8.0, containing 10% acetonitrile [v/v]) was added followed by 20 μl of 10 mM cyclization linker in acetonitrile (2 equiv.). The final concentrations in the reaction were 1 mM peptide, 2 mM cyclization linker, 60 mM

ammonium bicarbonate buffer and 35% acetonitrile. The plate was covered with a foil and the reaction incubated for 2 h at RT.

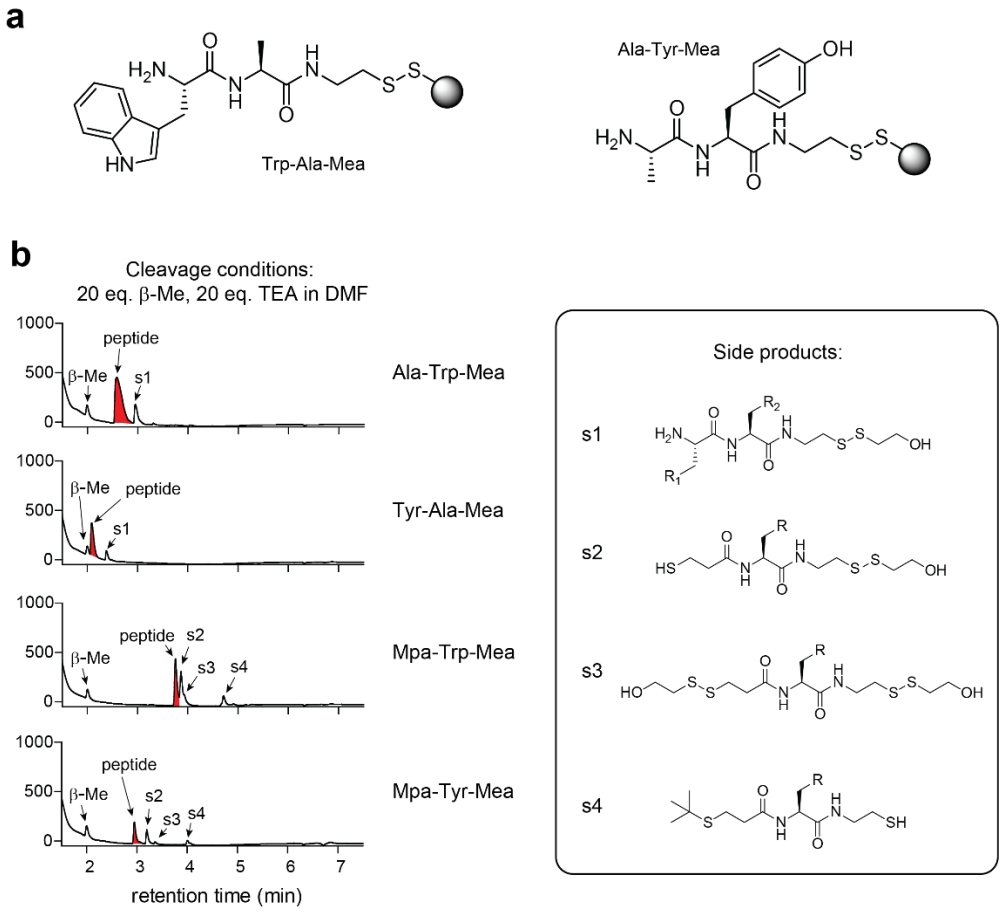
Quenching of linker reagents in cyclization reactions

After completion of the cyclization reaction, 4 μl of 150 mM β-Me in acetonitrile (0.6 μmol, 6 equiv. relative to the peptide) was added to the reaction mixture and incubated for 1 h at RT. The solvent (MeCN), buffer (bicarbonate) and excess β-Me were removed by centrifugal vacuum evaporation using Christ RVC 2-33 CDplus IR instrument. Samples were centrifuged at 0.1 mbar, 40 °C and at 400 × *g* (1750 rpm in a Christ 124700 rotor with 124708 plate holder inserts, radius = 10.5 cm). The quantity of peptide expected was so low that it could not be expected to be seen by eye, which was the case.

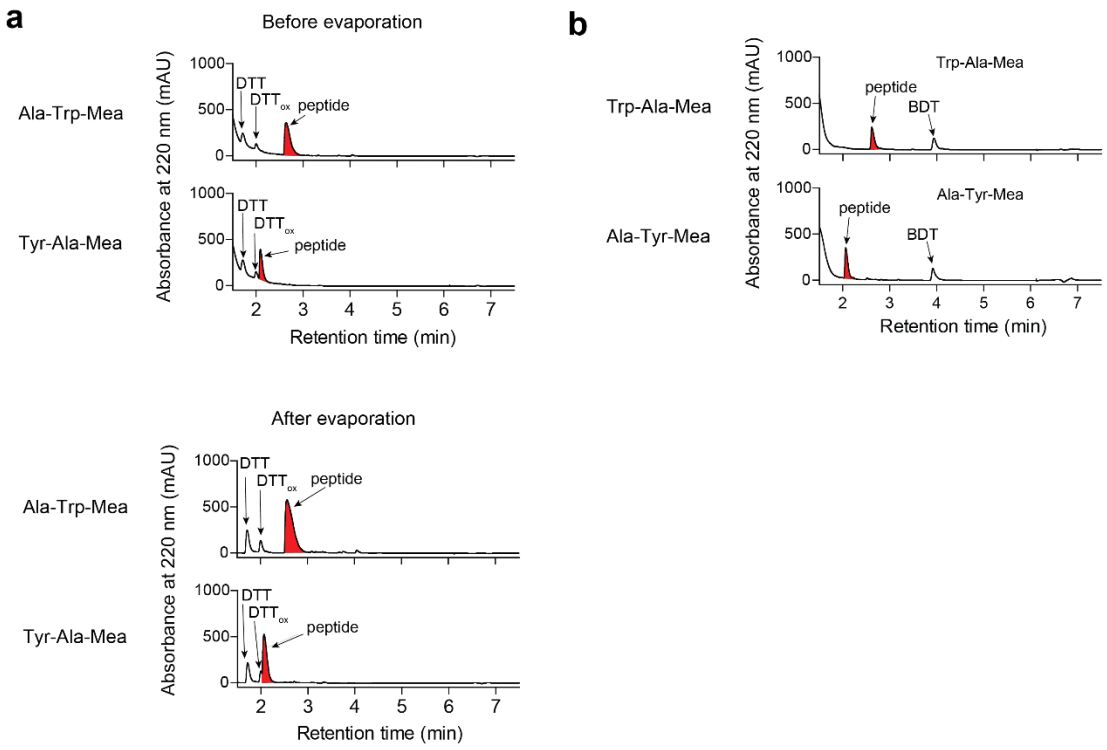
Concentration determination

The ThermoScientific NanoDrop 8000 spectrophotometer was blanked with milliQ H_2O. A baseline was measured at 280 nm with a mixture of 10 μl of cleavage mixture (250 mM TEA in DMSO for peptides released via cyclative release, and 100 mM TEA and 100 mM BDT in DMF for peptides released via reductive release) added to 90 μl DMSO. The average of three measurements was considered as a baseline value. For the concentration determination of peptide stocks, 1 μl of the peptide stock in the DMF solution obtained after the reductive release step was added to 9 μl of DMSO. The sample was measured three times, and the average was corrected by subtracting the baseline absorbance value. The extinction coefficients applied were ε_0(Trp)=5500 cm^{-1} M^{-1} for tryptophan containing sequences and ε_0(Tyr)=1490 cm^{-1} M^{-1} for tyrosine containing sequences. As BDT absorbs at 280 nm too, care was taken to ensure that the peptide concentrations measured were sufficiently high so the sample absorbance was several fold higher than the blank absorbance.

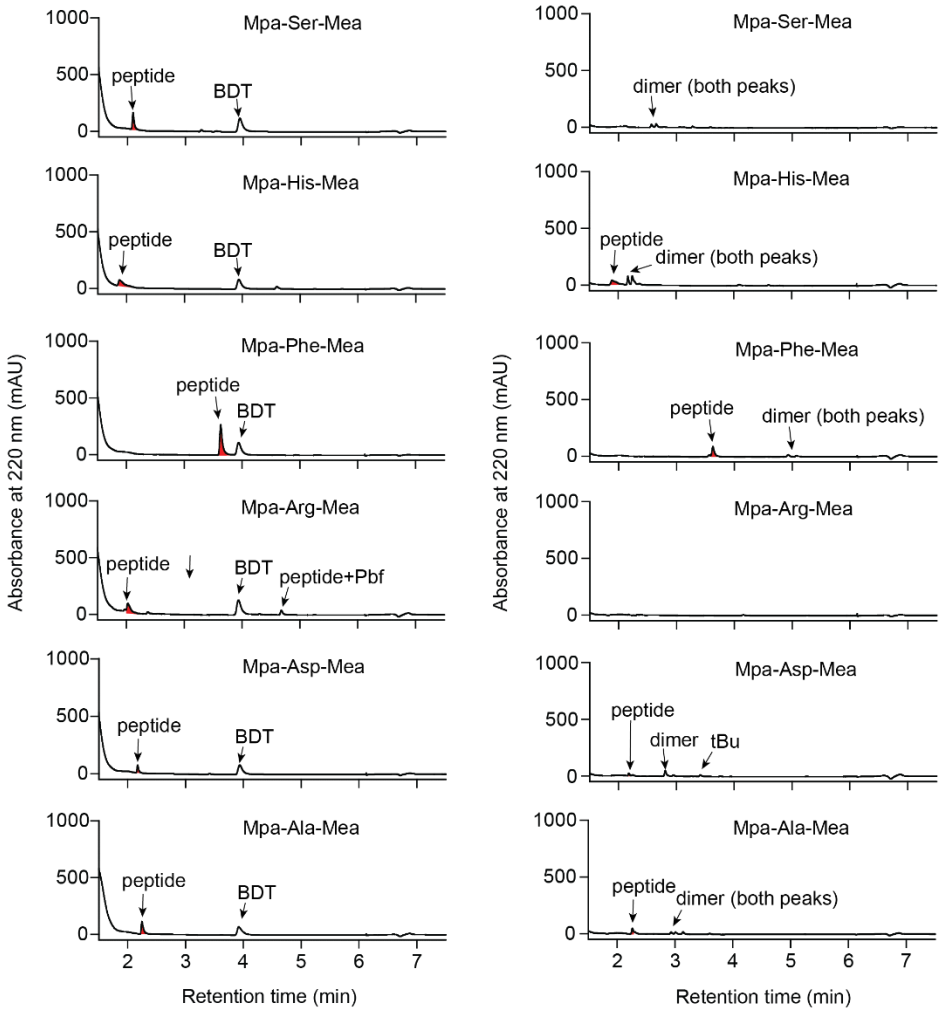
SUPPLEMENTARY FIGURES



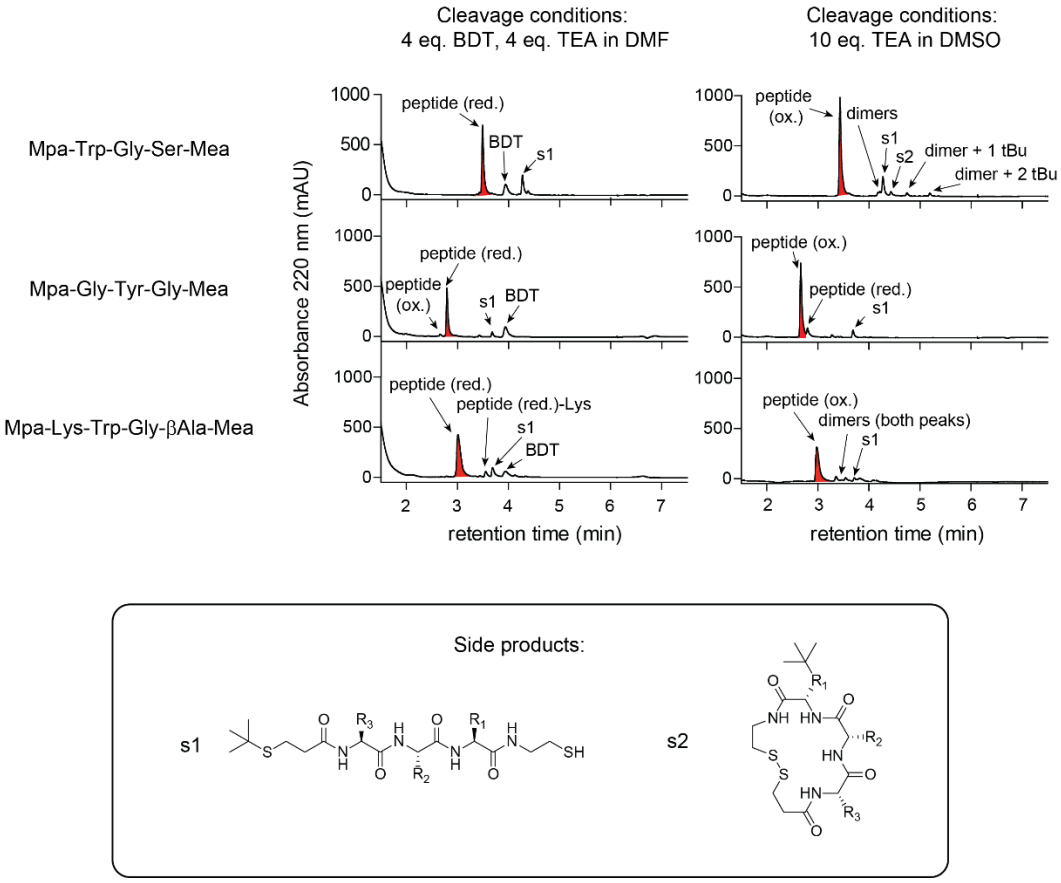
Supplementary Figure 1. SPPS of model peptides and release of disulfide-linked peptides from solid phase by β-Me. (a) SPPS of model peptides Ala-Trp-Mea and Tyr-Ala-Mea. (b) Reductive release by β-Me. The resin was incubated with 200 μl of DMF containing 500 mM β-Me (100 μmol, 20 equiv.) and 500 mM TEA (100 μmol, 20 equiv.) and incubated at room temperature overnight.



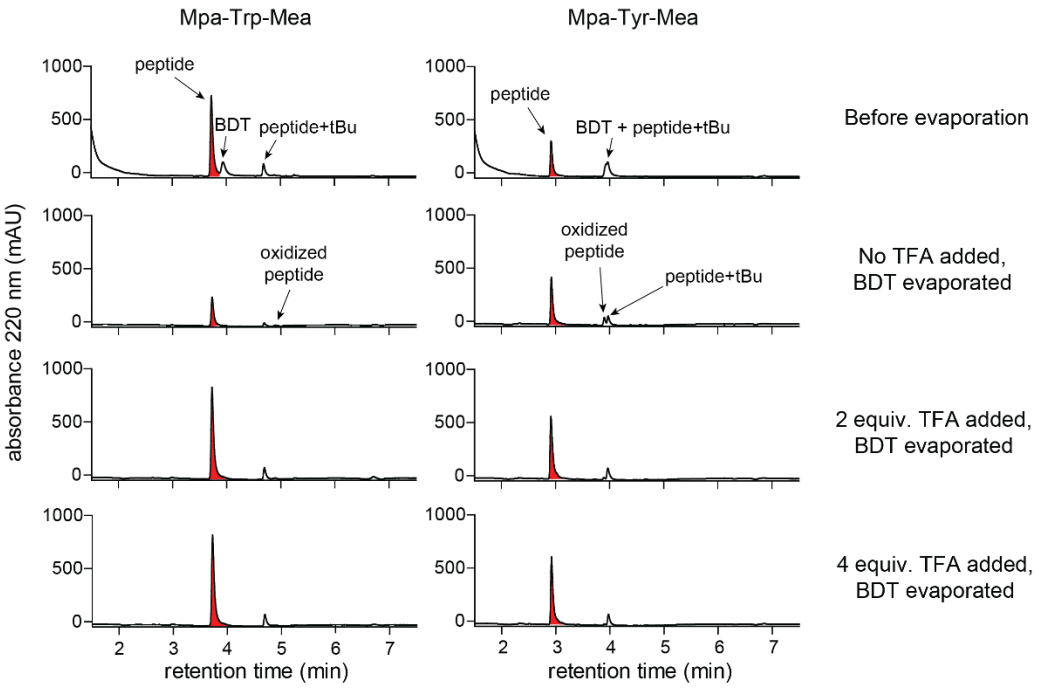
Supplementary Figure 2. Reductive release by DTT and BDT. (a) Release of disulfide-linked peptides from solid phase by 4 equiv. DTT and 4 equiv. TEA in DMF. The peptides were analyzed before (top) and after (bottom) evaporation of volatile agents by vacuum centrifugation. The resin was incubated with 200 μl of DMF containing 100 mM TEA (20 μmol, 4 equiv.) and 100 mM DTT (20 μmol, 4 equiv.) overnight at room temperature. (b) Release by BDT as shown in Figure 1c, tested with two additional peptides.



Supplementary Figure 3. Synthesis and release of diverse short dithiol peptides. Comparison of yields obtained by reductive release (left) and cyclative release (right). The peaks of the desired peptides are highlighted in red.



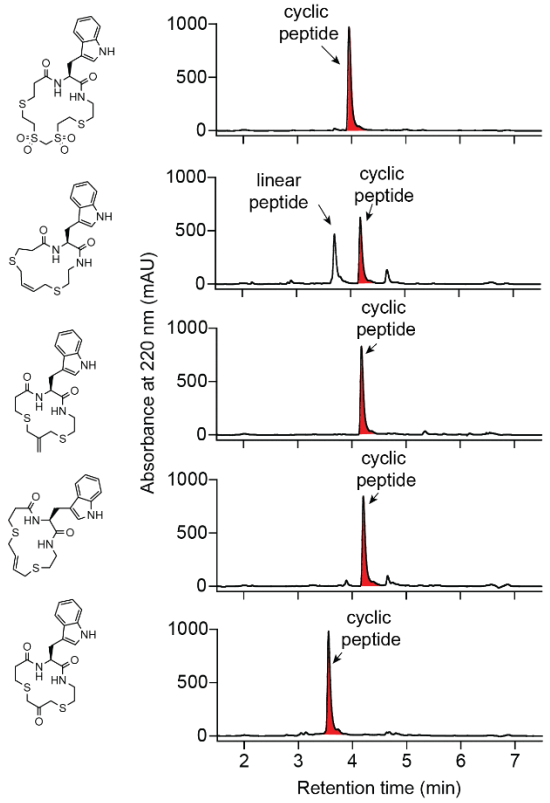
Supplementary Figure 4. Control peptides for cyclative disulfide release form solid phase. For reductive release, the resin was incubated with 200 μl of DMF containing 100 mM TEA (20 μmol, 4 equiv.) and 100 mM BDT (20 μmol, 4 equiv.), overnight at room temperature. For cyclative release, the resin was incubated with 200 μl DMSO containing 250 mM TEA (50 μmol, 10 equiv.) overnight at room temperature.



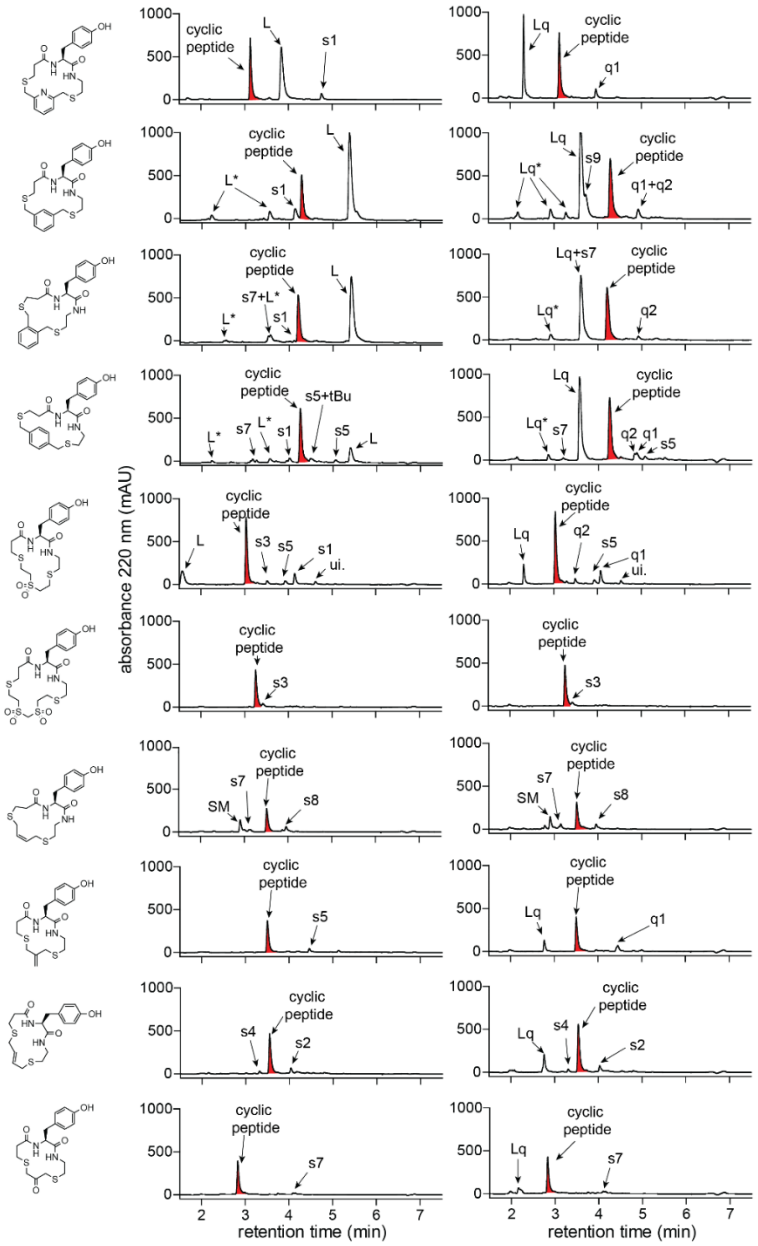
Relative peak areas

Peptide	TFA equiv. (rel. to base)	Peptide	Peptide+tBu	Peptide (ox)	Other
Mpa-Trp-Mea	Before evap.	87.9	11.2	0.3	0.7
	0	89.8	8.4	1.9	-
	2	88.7	9.6	0.3	1.4
	4	89.8	9	0.1	1.1
Mpa-Tyr-Mea	Before evap.	nd	nd	nd	nd
	0	74.9	14.4	9.3	1.4
	2	82.9	14.1	1.8	1.3
	4	85.9	12.3	1	0.8

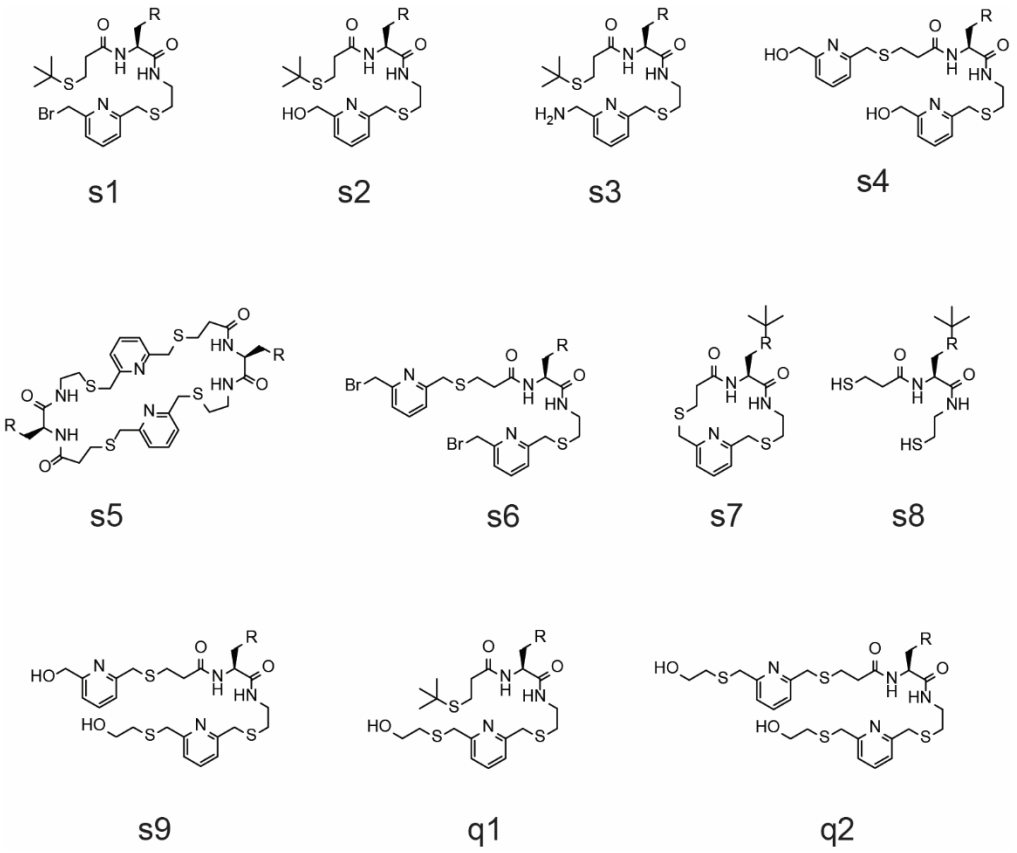
Supplementary Figure 5. Prevention of back-oxidation by addition of TFA prior to centrifugal vacuum evaporation. After reductive release, 0, 2 or 4 equiv. TFA were added to the peptide stocks as a 1% [v/v] (135 mM) solution in water. BDT was then removed by centrifugal vacuum evaporation at 0.1 mbar, 30 °C and at 400 × *g* for one hour.



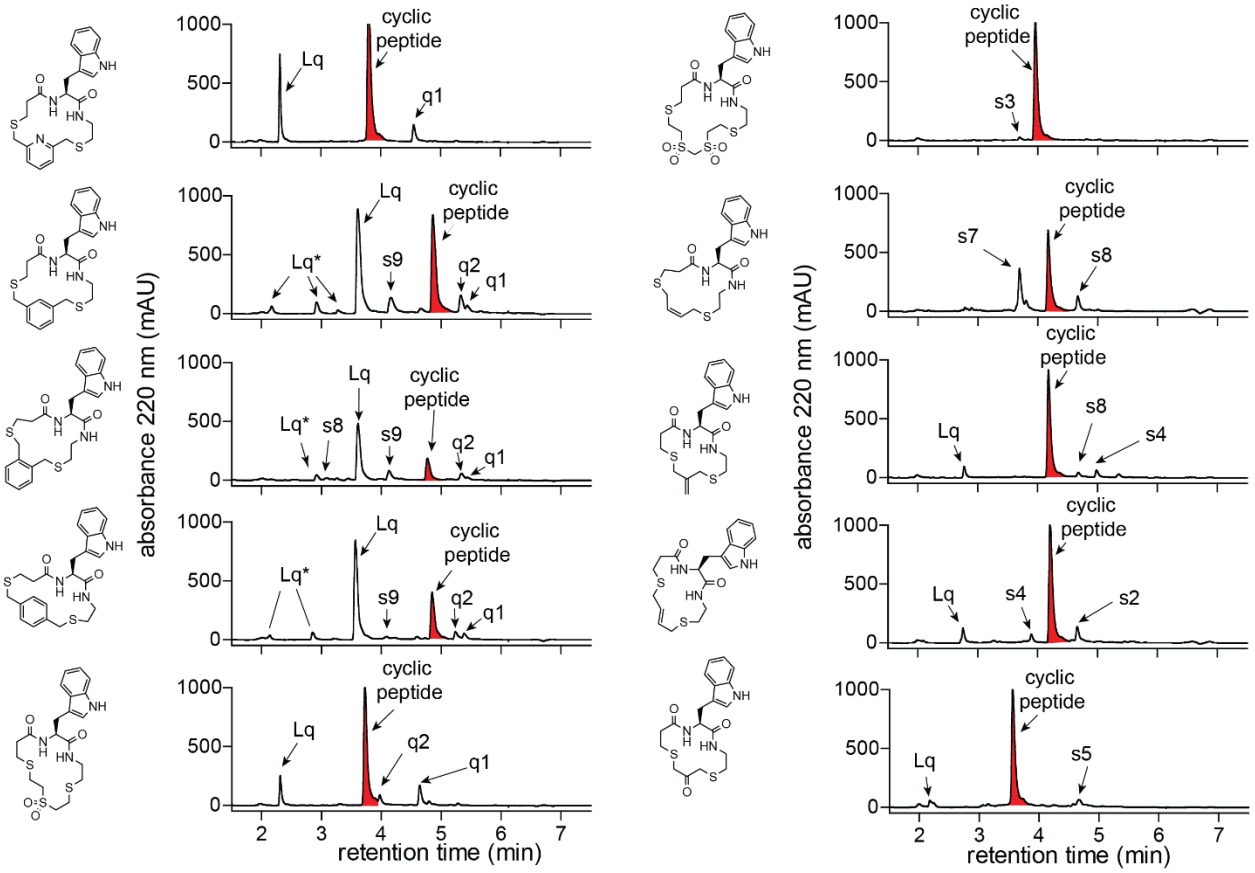
Supplementary Figure 6. Cyclization of dithiol peptide Mea-Trp-Mpa by bis-electrophile reagents **6**–**10**. The desired cyclic products are highlighted in red. L = bis-electrophile cyclization reagent. s1 = side-product tert-butyl-peptide-linker (structure shown in Supplementary Figure 8).



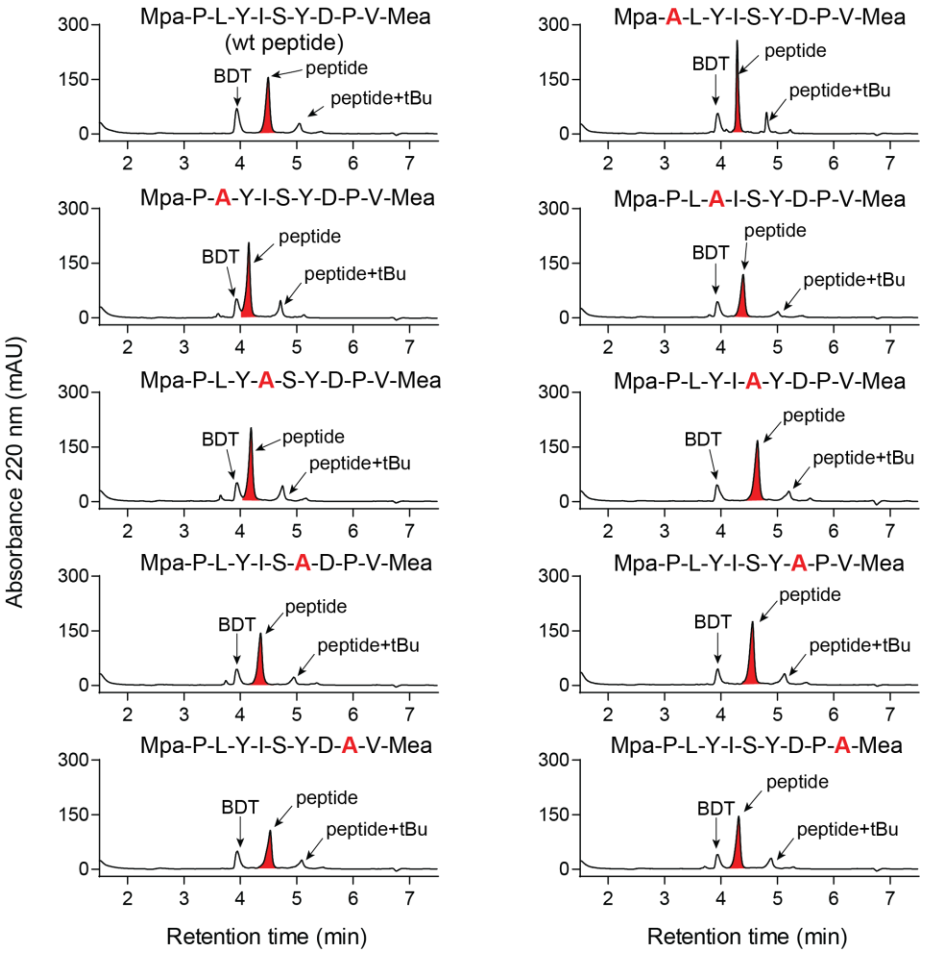
Supplementary Figure 7. Cyclization reactions with Trp and Tyr peptides. (a) Chemical structures of macrocycles and HPLC chromatographic analysis of the cyclization reactions before (left) and after (right) quenching of the bis-electrophile reagents with βMe. The desired cyclic products are highlighted in red. Side products s1 to s9 and q1 to q2 are shown in Supplementary Figure 7. L = bis-electrophile cyclization reagent. L* is hydrolyzed L. Lq = bis-electrophile cyclization reagent reacted with βMe. Lq* is hydrolyzed Lq.



Supplementary Figure 8. Structures of side products indicated in Supplementary Figures 6 and 8.



Supplementary Figure 9. Cyclization reactions with Trp peptides and quenching with βMe. The desired cyclic products are highlighted in red. Side products s2 to s9 and q1 to q2 are shown in Supplementary Figure 7. L = bis-electrophile cyclization reagent. L* is hydrolyzed L. Lq = bis-electrophile cyclization reagent reacted with βMe. Lq* is hydrolyzed Lq.



Supplementary Figure 10. Synthesis of longer peptides, tested with sequences based on the phage display-selected KRAS-specific peptide KRpep-2a. HPLC chromatograms of linear peptides containing the core sequence of KRpep-2a (wt peptide) and alanine mutants thereof.