A cyclative release strategy to obtain pure cyclic peptides directly from solid phase

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ABSTRACT

The synthesis of large numbers of cyclic peptides — required for example in screens for drug development — is currently limited by the need of chromatographic purification of individual peptides. Herein, we have developed a strategy in which cyclic peptides are released from solid phase in pure form and do not need purification. Peptides with an N-terminal thiol group are synthesized on solid phase via a C-terminal disulfide linker, their sidechain protecting groups are removed while the peptides remain on solid phase, and the peptides are finally released via a cyclative mechanism by addition of a base that deprotonates the N-terminal thiol group and triggers an intramolecular disulfide exchange reaction. The method yields disulfide-cyclized peptides, a format on which many important peptide drugs such as oxytocin, vasopressin, and octreotide are based. We demonstrate that the method is applicable for facile synthesis in 96-well plates, and allows for synthesis and screening of hundreds of cyclic peptides.

INTRODUCTION

Cyclic peptides have received much attention in recent years for their ability to target challenging proteins to which small molecule ligands are difficult or impossible to obtain. Currently, more than 40 cyclic peptides are approved as drugs, and more than 100 are being evaluated at different stages in clinical trials.[1] The development of peptide drugs typically involves multiple iterative cycles of synthesizing dozens to hundreds of variants to improve key properties such as binding affinity, specificity, stability, pharmacokinetic properties, etc., and thus requires the preparation of large numbers of peptides. Due to the typically low-yielding macrocyclization reactions,[2] and the presence of reagents and scavengers added for peptide release and sidechain deprotection, the chromatographic purification of each individual peptide is generally required. Even in a semi-automated fashion, this purification is expensive and time consuming, and limits the number of cyclic peptide variants that can be synthesized.

Herein, we aimed at developing an approach in which essentially pure cyclic peptides are released from solid phase, and that is compatible with synthesis in 96-well plates, in order to access large libraries of pure cyclic peptides that can readily be screened. We proposed the approach shown in Figure 1a, in which peptides bearing two thiol groups at their two ends, and being disulfide-linked to the solid phase via the C-terminal thiol, are synthesized on solid phase. We envisioned that all protecting groups would be removed while the peptides are on solid phase, allowing efficient elimination of protecting groups and reagents by bead washing, and that the peptides would be released in a disulfide-exchange reaction during which the peptides are cyclized. We proposed that this cyclative release could be initiated by deprotonating the N-terminally located thiol group, which would attack the disulfide bridge in an intramolecular fashion. The release mechanism would ensure that only cyclic peptide is released, omitting contamination of the product with linear peptide.

To our knowledge, the proposed strategy of cyclative disulfide release is completely new and has not been reported. Strategies that come closest to our plans are the oxidative release of thioether-immobilized peptides,[3,4] but we consider them as not suitable for library generation due to the low yields, dimeric side products, and the presence of oxidants in the eluted product that would need removal by purification. Cyclative peptide release strategies were developed before, using elegant strategies and yielding peptide cyclized via different chemical bonds.[5] Strategies based on

cyclization from thioester linkers,[6] olefin linkers for ring-closing metathesis,[7] and Dawson's MeDbz linker for native chemical cyclization[8,9] were used to synthesize and screen peptide libraries, but the released peptides still required chromatographic purification prior to screening.

RESULTS AND DISCUSSION

In order to test the strategy proposed in Figure 1a for cyclative release of peptides from solid phase by intramolecular disulfide exchange, we synthesized the test peptide Mpa-Gly-Gln-Trp-Mea, with Mpa representing 3-mercaptopropionic acid (cysteine without the amino group) and Mea representing 2-mercaptoethylamine (cysteamine; cysteine without carboxylic acid), that was linked via a disulfide bridge to resin (Figure 1b). For this synthesis, we first conjugated the Mea building block to thiol-functionalized resin as described in the Supplementary Results, and then coupled the amino acids and Mpa using standard Fmoc chemistry. In order to test the synthesis, on-resin side chain deprotection, and cyclative release in different microenvironments, we tested five resins: two polar polyethylene glycol (PEG) resins (resins 1 and 2), one polar PEG-modified polystyrene (PS) resin (resin 3), and two apolar PS resins (resins 4 and 5) (5 μmol scale; Figure 1b and Supplementary Table 1). Weighing of the resins before and after synthesis indicated that peptides were efficiently synthesized on all resins (Supplementary Table 2). Sidechain deprotection followed by disulfide bond reduction released peptide with the correct mass (Supplementary Figure 1, and Supplementary Table 3 and 4).

Next, we tested whether the peptide can be released from the resin via the anticipated cyclization mechanism by deprotonating the sulfhydryl group at the N-terminal end of the peptide. Incubating the resins in DMSO with the base DIPEA (150 mM) led to highly efficient release for the resins 4 and 5 (Figure 1d and Supplementary Table 5). The yields of peptide in the eluate were 1.05 ± 0.25 μmol (resin 4) and 2.2 ± 0.2 μmol (resin 5), and thus not too far from the maximal amount that could be expected based on the resin loading (5 μmol), and far more sufficient than required for screening. The two high-yielding resins were apolar (polystyrene) and the three resins that did not work were polar (PEG), suggesting that the polarity of the solid phase may influence the disulfide exchange reaction, but we did not further study potential mechanisms. Equally important as the high yields were the high concentrations of 5.1 mM (resin 4) and 11.6 mM (resin 5), that allowed convenient use of the peptide for high-throughput screening without concentration or solvent or base removal. For example, stocks in DMSO may be diluted 1000-fold for testing the cyclic peptides at 10 μM, which is a typical screening concentration, leaving negligible amounts of DMSO (0.1%) and DIPEA (150 μM) in the assay that are unlikely to affect biological assays. Liquid chromatography-mass spectrometry (LC-MS) analysis of the products showed high purities of 95 ± 4% (resin 4) and 93 ± 1% (resin 5) for the disulfide-cyclized peptide (Figure 1d and Supplementary Figure 2). The only side product observed was found in small quantities (6% on

average) and corresponded to a cyclic peptide dimer, most likely formed by disulfide exchange-mediated transfer of one peptide to a neighboring one on the resin and the subsequent cyclative release of a cyclic dimer.

To assess the substrate scope of the cyclative disulfide release strategy, we synthesized four peptides with the sequence Mpa-Gly-Ala-Xaa-Mea, where “Xaa” are amino acids that imposed various levels of rigidity into the peptide backbone (Trp, Amb, Pro, Nip; Figure 2a). HPLC analysis of the products obtained by base-induced cyclative release showed a dominant peak for each one of the four peptides, and MS analysis confirmed that these main products were the desired cyclic peptides. Yields ranged from 54—100% (13.5 to 26 μmol), as calculated via absorbance and weight after lyophilization (Supplementary Table 6). For all four peptides, only small quantities of a limited number of side products were observed (Figure 2b and Supplementary Table 6). The main side products were cyclic dimers, eluting as two close peaks, most likely corresponding to the head-to-head/tail-to-tail dimer and the head-to-tail version. Linearization of the products with TCEP and subsequent HPLC analysis showed linear peptide products with purities of 96, 96, 100, and 92% for peptides 1—4, respectively (Figure 2b, right chromatograms). The TCEP-linearization experiment suggests that dimeric peptides can be reduced and then re-cyclized at low concentrations to favor the intramolecularly cyclized monomer.

To see if shorter peptides could be generated following the cyclative release strategy, which is important to test due to the strain of a smaller ring, we repeated the experiment with four new peptides that were one amino acid shorter than in the previous experiment (Supplementary Figure 3a). These four peptides with the format Mpa-Ala-Xaa-Mea (Xaa = Trp, Amb, Pro, Nip) were efficiently released, and the main peak in the HPLC profile was the desired cyclic peptide (Supplementary Figure 3b and Supplementary Table 7). The fraction of cyclic dimer was slightly higher overall, probably due to a less efficient circularization resulting from conformational constraints of the short backbones.

To determine whether the cyclative disulfide release strategy can be applied for library synthesis and screening, we designed and synthesized cyclic peptides in a 96-well plate (5 μmol scale; Supplementary Figure 4). We prepared 96 random disulfide-cyclized peptides with three different formats, which contained three random amino acids (“Xaa”) flanked by Mpa and Mea (Figure 3a).

Because variation of the peptide backbone is critical for generating structurally diverse macrocycle libraries, two of these random amino acids in each peptide were selected from four structurally diverse α-, β-, and γ-amino acids to generate diverse cyclic peptide backbones (amino acids I to IV; Figure 3a). The third random amino acid was Trp or Tyr, which allowed for quantification of peptide yields by absorption measurement at 280 nm. Cyclative release of the peptides and subsequent absorption measurement showed a high average peptide yield (2.7 μmol), a high average concentration (13.3 mM) and a narrow concentration distribution for 90 of the peptides between 8.9 mM (1.5-fold below average) and 20 mM (1.5-fold above average). Three of the peptides were not synthesized or released at all. Analysis of 12 randomly selected cyclic peptides by LC-MS showed excellent purities (Supplementary Figure 5 and 6). All amino acid building blocks used for the synthesis were compatible with the synthesis of linear peptides immobilized via a disulfide bridge as well as with the cyclative disulfide release strategy.

We performed a screen against thrombin, a target for developing anticoagulation therapeutics, using a compound concentration of approximately 10 μM. The most active peptide reduced thrombin activity by around 50%, which was remarkable considering that the peptide did not contain amino acids Arg or Lys that form key interactions with the thrombin substrate recognition pocket S1 (Figure 3c). Repetition of the screen identified the same cyclic peptide as the most active hit (green dots in Figure 3c). The HPLC-purified disulfide-cyclized peptide Mpa-Tyr-II-Pro-Mea inhibited thrombin with a K_i of 13 ± 1 μM (Supplementary Figure 7). Additionally, it is important to note that the DMSO and DIPEA, present after release, did not affect the biological screen.

We finally tested if cyclic peptoids, which could be attractive for the development of membrane-permeable macrocycles, can be synthesized with the new method (Figure 4 and Supplementary Figure 8a). As described in detail in the Supporting Information, most cyclic peptoids of a 342-member library were obtained in high yields (2.2 μmol average yield and 11 mM average concentration), and they showed a narrow yield and concentration distribution (Figure 4, Supplementary Figure 9 and 10), which should allow direct screening of the macrocyclic compounds without determining and normalizing the concentrations. An analysis of the physicochemical properties important for membrane permeability (MW, cLogP, polar surface area, number H-bond donors) and a comparison with those of 34 orally available macrocycle

drugs or drug candidates[10] suggest that some of the peptoids produced by the cyclative release strategy could potentially be orally available (Supplementary Figure 8b).

In summary, we have addressed the bottleneck in producing cyclic peptide libraries, being the purification step, by developing a cyclative peptide release strategy based on a disulfide exchange reaction that releases highly pure disulfide-cyclized peptides directly from the solid support. Because of the high purity and a volatile base as cleavage reagent, which can be removed by evaporation, these peptides can be readily screened using bioassays without prior purification. Importantly, the yields of the library components containing different building blocks and sequences were narrowly distributed, permitting screening without the need to determine or adjust the concentrations of hundreds of compounds. We have shown that the approach is applicable for the generation of libraries comprising hundreds of peptides, which can be synthesized and screened in only a few days. Our rapid and facile method allows for the access of chemical space that was previously difficult to access. Importantly, the method can be applied to peptides, peptoids, and in principle any molecule linked via a disulfide bond to solid phase and containing another thiol, allowing for even larger increases in chemical diversity. As a next step, we intend to further increase our synthetic throughput and screen targets relevant for the treatment of disease.

METHODS

Synthesis of model peptide on different resins

Aminomethyl ChemMatrix resin (100 mg, 100 μmol, 1.0 mmol g^{-1}; Sigma-Aldrich), NovaPEG amino resin (189 mg, 100 μmol, 0.53 mmol g^{-1}; Novabiochem), HypoGel 200 NH_2 (111 mg, 100 μmol, 0.9 mmol g^{-1}; Sigma Aldrich), Aminomethyl polystyrene resin (71.9 mg, 100 μmol, 1.39 mmol g^{-1}; Aapptec) and Polystyrene A SH (105 mg, 100 μmol, 0.95 mmol g^{-1}; Rapp Polymer) were introduced into five different 5 ml polypropylene synthesis columns (MultiSyntech GmbH, V051PE076) and SPPS was performed manually.

To all resins except to Polystyrene A SH, the thiol source *S*-trityl-3-mercaptopropionic acid (Trt-MPA) was coupled by reacting the carboxylic acid of Trt-MPA with amino groups on the resins. For this, the resins were pre-washed as follows: 2 × 3 ml MeOH, 2 × 3 ml DCM, 2 × 3 ml 1:99 TFA/DCM (v/v), 2 × 3 ml 1:19 DIPEA/DCM (v/v), 2 × 3 ml DCM. Immediately before coupling the Trt-MPA, the amino resins were washed with 2 × 3 ml DMF. Trt-MPA was coupled twice using Trt-MPA (150 mM, 3 equiv.), HATU (150 mM, 3 equiv.) and DIPEA (300 mM, 6 equiv.) in 2 ml DMF. The two consecutive coupling reactions were performed for one hour at room temperature under rotation (20 rpm). After the coupling, the resins were washed with 3 × 3 ml DMF. Subsequently, a capping step was applied using 2 ml capping solution containing 5:6:89 Ac_2O/2,6-lutidine/DMF (v/v/v) for 5 min. After the capping, the resins were washed with 3 × 3 ml DMF. The Trt group was deprotected using 2 × 3 ml of 10:1:89 TFA/TIS/DCM (v/v/v) for 2 × 1 hour at room temperature under rotation (20 rpm) followed by washing with 3 × 3 ml DCM.

Before the disulfide exchange reaction, all the resins were washed with 3 × 3 ml 3:7 MeOH/DCM (v/v). *S*-(2-pyridylthio)cysteamine hydrochloride (98.0 mg, 0.44 mmol, 4.4 equiv.) in 3.5 ml of 3:7 MeOH/DCM (v/v) was added to the resins followed by the addition of DIPEA (76.6 μl, 0.44 mmol, 4.4 equiv.). The disulfide exchange reactions were performed by incubation for three hours at room temperature. The resins were washed with 3 × 3 ml 3:7 MeOH/DCM v/v, and 3 × 3 ml DMF.

Afterwards, the different peptide building blocks were installed in the following order using SPPS: Trp, Gln, Gly, and Trt-MPA. All couplings were carried out twice for each building block using protected amino acid/carboxylic acid derivative (150 mM, 3 equiv.), HATU (150 mM, 3 equiv.),

and DIPEA, (300 mM, 6 equiv.) in 2 ml DMF. Coupling reactions were performed for one hour at room temperature under rotation (20 rpm). After each double-coupling, 2 ml capping solution (5:6:89 Ac_2O/2,6-lutidine/DMF [v/v/v]) was applied for five minutes at room temperature under rotation (20 rpm). After each coupling and capping step, the resins were washed with 3 × 3 ml DMF. Fmoc protecting groups were removed with 2 × 3 ml 1:4 piperidine/DMF (v/v) for five minutes. The resins were washed with 3 × 3 ml DMF and 3 x 3 ml DCM. The extent of coupling was qualitatively assessed after each step using a Kaiser and/or Ellman's test. At the end of the synthesis, the resins were washed with DCM. The residual DCM was allowed to evaporate in air for four hours, and the samples were placed under vacuum for drying until the measured mass stopped decreasing (24 hours). The dry weights of the resins were measured after the synthesis. Five μmoles of each resin were transferred to wells of 96-well plates (Orochem, OF 1100). The weight transferred for each resin/peptide is shown in the last row of the Supplementary Table 2.

Amino acid sidechain deprotection

For side chain removal, the bottom of the 96-well synthesis plate was sealed by pressing the plate onto a soft 6 mm thick ethylene-vinyl acetate foam pad (Rayher Hobby GmbH, 78 263 01), and the resin in each well was incubated with around 500 μl of 38:1:1 TFA/TIS/ddH_2O (v/v/v). The plates were covered with a polypropylene adhesive seal, then weighed down by placing a weight (1 kg) on top to ensure that no leakage occurred. After 1.5 hours, the synthesis plates were placed onto 2 ml deep-well plates, and the TFA mixture was allowed to drain. The wells were washed three times with approximately 500 μl of DCM (added with syringe), then allowed to air dry for three hours.

Cyclative release

Plates were pressed into foam pads as described above to plug the openings, and 200 μl of 150 mM DIPEA in DMSO (6 equiv.) were added to each well. The plates were sealed with an adhesive foil, weighed down (1 kg), and left overnight. The next day, the synthesis plates were placed onto 2 ml deep-well plates and centrifuged at around 200 g (1000 rpm with a Thermo Heraeus Multifuge 3L-R centrifuge) for one minute to collect the released cyclic peptides in DMSO.

Preparation of polystyrene-S-S-cysteamine resin for library synthesis

The following procedure was applied to prepare polystyrene-S-S-cysteamine resin needed for the synthesis of 4 × 96 peptides at a 5 μmol scale in four 96-well plates. Into each of four 20 ml plastic syringes (CEM, 99.278) was added 589 mg resin (Rapp Polymere Polystyrene A SH resin, 200-400 mesh, 0.85 mmol g^{-1} loading), corresponding to a 0.5 mmol scale per syringe, and a 2 mmol scale in total. The resin of each syringe was washed with 15 ml of DCM, then swelled in 15 ml of 3:7 MeOH/DCM v/v for 15 minutes. 2-(2-pyridinyldithio)-ethanamine hydrochloride (1.96 grams, 8.8 mmol, 4.4 equiv.) was dissolved in 21.12 ml of MeOH, then 49.28 ml of DCM and 1.53 ml of DIPEA (8.8 mmol, 4.4 equiv.) were added. A volume of 17.7 ml of this solution was pulled into each syringe and the syringes shaken at room temperature for three hours. After this time, the 2-(2-pyridinyldithio)-ethanamine solutions were discarded, and the resins were washed with 2 × 20 ml 3:7 MeOH/DCM v/v, then 2 × 20 ml DMF. The resins were combined into a single syringe as a suspension in DMF and washed with 11.8 ml of 1.2 M DIPEA solution in DMF for five minutes to ensure that all amines were neutral. This solution was discarded, and the resin was washed with 2 × 20 ml DMF, 4 × 20 ml DCM, then kept under vacuum overnight to yield a free-flowing powder.

Peptide library synthesis in 96-well plates

Automated solid-phase peptide synthesis was performed on an Intavis Multipep RSi synthesizer. To a 50 ml tube was added 565 mg of polystyrene-S-S-cysteamine resin (0.48 mmol S-S-cysteamine assuming that thiol groups were quantitatively modified with cysteamine) and 20 ml of DMF. The tube was shaken to ensure the resin was uniformly suspended, and 200 μl (5.88 mg resin, 5 μmoles) were transferred to each well of a 96-well solid phase synthesis plate (Orochem, OF 1100). The resin was washed with 6 × 150 μl DMF. Coupling was performed with 53 μl of amino acids (500 mM, 5.3 equiv.), 50 μl HATU (500 mM, 5 equiv.), 12.5 μl of *N*-methylmorpholine (4 M, 10 equiv.), and 5 μl *N*-methylpyrrolidone. All components were premixed for one minute, then added to the resin (one hour reaction, no shaking). The final volume of the coupling reaction was 120.5 μl and the final concentrations of reagents were 220 mM amino acid, 208 mM HATU and 415 *N*-methylmorpholine. Coupling was performed twice, then the resin was washed with 6 × 225 μl of DMF. Fmoc deprotection was performed using 120 μl of 1:5 piperidine/DMF v/v for 5 minutes, and was performed twice. The resin was washed with 8 × 225 μl DMF. At the end of the peptide synthesis, the resin was washed with 2 × 200 μl of DCM.

Peptoid library synthesis

The peptoid library was synthesized in 96-well plates as the peptide library. For the incorporation of peptoid building blocks, 62 μl bromoacetic acid in DMF (1 M, 12.4 equiv.) or 3-(bromomethyl)benzoic acid in DMF (1 M, 12.4 equiv.) and 63 μl of *N,N*-diisopropylcarbodiimide in DMF (1 M, 12.6 equiv.) were pre-incubated for one minute, added to the wells, and incubated for one hour without shaking. The reactions were repeated once and the resin washed with 5 × 225 μl DMF. Next, 125 μl of amine in DMF (2 M in DMF, 50 equiv.) was added to the wells and the reactions incubated for two hours. The wells were washed with 5 × 225 μl DMF.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Results section describing the synthesis of the cyclic peptide library with small polar surface. Materials and methods for routine procedures applied in this study. Tables providing additional experimental data and raw data. Figures providing HPLC chromatograms of cyclic peptides, a step-by-step protocol for the cyclative release method, and a description of the cyclic peptide library with small polar surface.

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Notes

The authors declare no competing financial interests.

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FIGURES

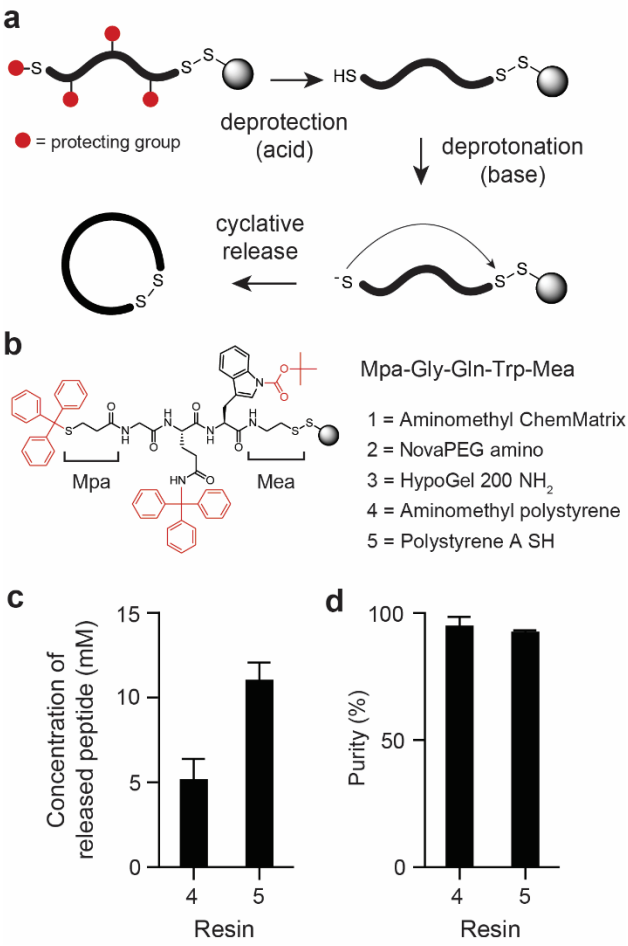


Figure 1. Cyclative disulfide release strategy. (a) Schematic representation of short peptides synthesized via a disulfide bridge on solid phase. Protecting groups (red) are removed on solid phase for an efficient separation of the cleavage reagents. Treatment with a base deprotonates the N-terminal thiol, which induces an intramolecular disulfide exchange to generate the cyclic product. (b) Chemical structure of test peptide Mpa-Gly-Gln-Trp-Mea disulfide-linked to a solid support and the commercial resins used. (c) Recovery of disulfide-cyclized peptide Mpa-Gly-Gln-Trp-Mea synthesized on resins 4 and 5 and released with 150 mM DIPEA in DMSO. Concentrations were determined by measuring absorbance at 280 nm. Reactions were performed in triplicate. (d) Purity of disulfide-cyclized peptide of panel c. Purity was determined by LC-MS, measuring the AUC of all species at 220 nm UV absorbance.

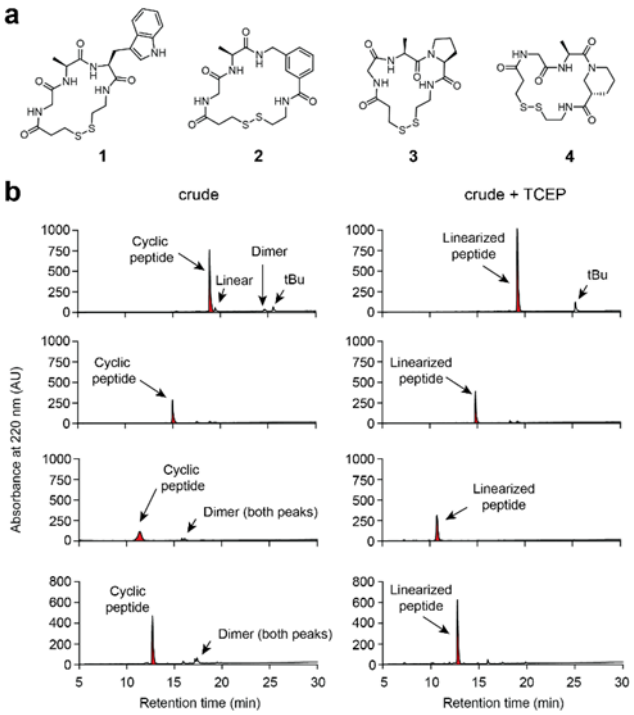


Figure 2. Cyclative release of peptides with variable sequences. (a) Chemical structures of the desired four cyclic peptides. The peptides are based on the linear sequence Mpa-Gly-Ala-Xaa-Mea, with “Xaa” being amino acids having varying conformational flexibilities in their backbone (Trp, Amb, Pro, Nip). (b) Analytical HPLC chromatograms of the crude peptide after cyclative release. The chromatograms on the right show the peptides after disulfide-bond reduction with TCEP. For impurities that were not identified, the mass is indicated (assuming that the species are singly charged). tBu = peptide with tBu group.

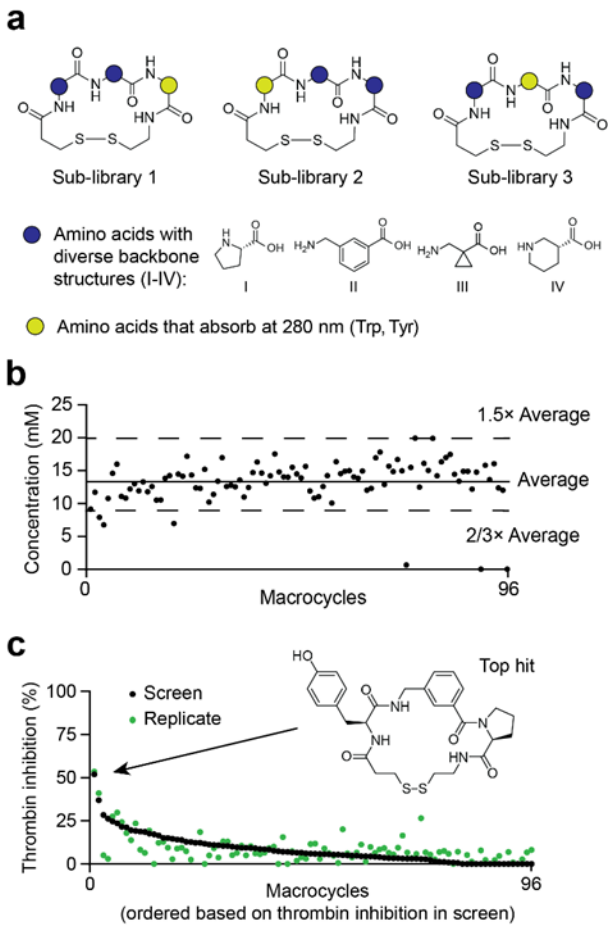


Figure 3. Library design, peptide recovery quantification by absorption, and thrombin inhibition of the cyclic peptide library. (a) Design of library comprising 96 different peptides and structures of unnatural amino acids used in the library. (b) Scatter plot of cyclic peptide concentrations (mM in DMSO) for the 96 synthesized cyclic peptides quantified by absorption at 280 nm. The average recovery, along with 1.5× and 0.67× of this value, is indicated on the chart. (c) Thrombin inhibition measured at an average cyclic peptide concentration of 11 μM. The peptides are ordered according to their thrombin inhibition activity in the first screen (black dots; highest to lowest activity). Green dots indicate thrombin inhibition for the same cyclic peptides measured in a second screen using the same conditions. The chemical structure of the most active inhibitor is shown (K_i = 13 ± 1 μM).

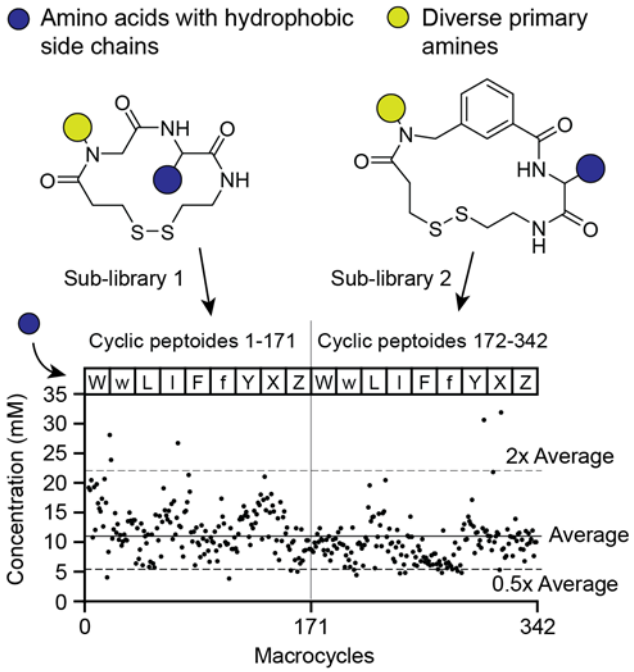
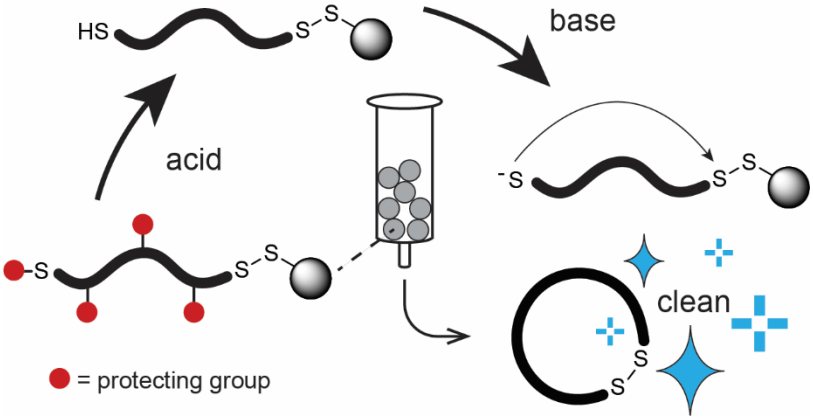


Figure 4. Library composed of 342 small cyclic peptoids. The scaffolds of the two sub-libraries are shown in the top and the concentrations of the 342 cyclic peptoids generated are shown in the graph below. The concentrations were determined by an Ellman's reagent-based assay. The cyclic peptoids in the graph are sorted first according to the scaffold type (sub-library 1 first), second according to the amino acids in the molecules (blue building block), and third by amine building blocks (yellow building block). The chemical structures of the amino acid building blocks (W, w, L, l, F, f, Y, X, Z) and the amines (N1–N19) are shown in Supplementary Figure 8.

TABLE OF CONTENTS FIGURE



SUPPORTING INFORMATION

A cyclative release strategy to obtain pure cyclic peptides directly from solid phase

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Contents:

Supplementary Results

Synthesis of short cyclic peptides with a small polar surface area

Regarding the development of cell permeable peptides, we tested whether smaller, *N*-substituted cyclic peptides could be synthesized by the new cyclative disulfide release strategy. *N*-substitution removes one H-bond donor from the peptide, thus increasing the chances of membrane permeability. In these peptides, one of four building blocks was *N*-substituted, which we reasoned should still cyclize efficiently due to the higher rotational flexibility around the peptide bond between Mpa and the "peptoid" amino acid, even with the shorter length of only four building blocks. To obtain the two cyclic peptide formats shown in Figure 4, the *N*-substituted amino acids, either glycine (sub-library 1) or 3-(aminomethyl)benzoic acid (sub-library 2), were introduced using the sub-monomer approach, by first appending bromoacetic acid or 3-(bromomethyl)benzoic acid and then adding a primary amine. We combinatorially synthesized a total of 342 peptides by using nine different amino acids, two sub-monomer building blocks, and 19 different primary amines (Supplementary Figure 8a). The majority of chosen amino acids and amines were not charged, and had a limited polarity to achieve cyclic peptides with physicochemical properties that resembled those of approved cell permeable or orally available macrocyclic drugs. Indeed, there was a good overlap between the 342 cyclic peptides and 34 orally available macrocyclic drugs or drug candidates when comparing the molecular weight, the calculated water/n-octanol partition coefficient (clogP), the number of H-bond donor groups, and the polar surface area (Supplementary Figure 8b).

Because most entries in the library did not contain Trp or Tyr, whose absorption at 280 nm form the established method for measuring peptide concentrations, we next established a procedure to determine concentration based on the presence of thiols. In brief, we incubated the released peptides with immobilized TCEP to reduce the disulfide bridge, transferred the reduced peptides to wells of a microtiter plate containing Ellman's reagent, and quantified the number of sulfhydryl groups by measuring the absorption of released 2-nitro-5-thiobenzoic acid (TNB) species at 412 nm. In parallel, we determined the concentrations of the 26 peptides that contained Trp or Tyr by measuring their absorbance at 280 nm and found that the concentrations determined based on the thiol groups correlated well with the values measured by UV absorption (Supplementary Figure 9). We found that 320 of the 342 library entries were obtained in high yields, with an average yield of 2.2 μmol (11 mM average concentration) and a narrow yield and concentration distribution (Figure 4), indicating an efficient release by the cyclative disulfide method despite the short peptide sequences and potential conformational constraints. Overall, the cyclic peptides containing *N*-substituted amino acid and having shorter macrocyclic rings were obtained in good yields showing that molecules with properties of oral drugs can be released well. LC-MS analysis of 26 sample peptides, half of which pertain to each sub-library and with all 19 different amine building blocks represented, showed a high purity of the desired peptides (85% average for 26 peptides; Supplementary Figure 10).

Supplementary Materials and Methods

General considerations

Unless otherwise noted, all reagents were purchased from commercial sources and used with no further purification. Solvents were not anhydrous, nor were they dried prior to use. All water was purified using a MilliQ Integral 5 water purification system (Merck).

Kaiser and Ellman's tests

For the Kaiser test, three droplets of each of the following solutions A, B and C were given into a vial containing a few test beads. The test mixture was gently warmed with a heat gun until a blue/purple coloration was visible. Solution A: 200 μM KCN in H_2O/pyridine (1:49), Solution B: 280 mM ninhydrin in 1-butanol, Solution C: 21 M phenol in 1-butanol. As a negative control, Polystyrene A SH resin was used (no coloration). As a positive control, Aminomethyl polystyrene resin was used (dark blue).

For the Ellman's test, a spatula tip of test beads were pre-swelled by adding THF (100 μl, 10 min) followed by the addition of 100 μl Ellman's reagent (5 mM in MeOH) and DIPEA (1 μl). The sample was further diluted with methanol (299 μl). A yellow coloration appears in presence of free thiols. As a negative control, Aminomethyl polystyrene resin was used (colorless). As a positive control, Polystyrene A SH resin was used (yellow coloration).

Reductive release

The bottom of the 96-well synthesis plate was sealed by pressing the plate onto a soft 6 mm thick ethylene-vinyl acetate foam pad (Rayher Hobby GmbH, 78 263 01). A volume of 200 μl of DMSO/H_2O (9:1, v/v) containing 25 μmol TCEP·HCl (5 equiv.) were added to the wells. The plate was covered with an adhesive aluminum seal, a weight (1 kg) was placed on top of it to prevent detachment of the plate from the ethylene-vinyl acetate pad and leakage of the solutions, and incubated overnight at room temperature. The plate was placed into a 96-deep well plate (Thermo Scientific, 278752) and the solutions transferred by applying vacuum. The concentration of peptide in the filtrates were determined by measuring absorption at 280 nm (Thermo Scientific, Nanodrop 8000) and calculated assuming that mainly Trp absorbs (A_{280}, $\varepsilon_{Trp,\ 280\ nm}$ = 5,500 $M^{-1}*cm^{-1}$, d = 0.1 cm).

LC-MS analysis

Peptides were analyzed by LC-MS analysis with a UHPLC and single quadrupole MS system (Shimadzu LCMS-2020) using a C18 reversed phase column (Phenomenex Kinetex 2.1 mm × 50 mm C18 column, 100 Å pore, 2.6 μM particle) and a linear gradient of solvent B (acetonitrile, 0.05% formic acid) over solvent A (H_2O, 0.05% formic acid) at a flow rate of 1 ml min^{-1}. Mass analysis was performed in positive ion mode.

Model peptides eluted in DMSO/DIPEA were diluted around 100-fold with water to reach a concentration of 100 μM, and 5 μl of the samples were injected and analyzed using a 0 to 60% gradient of acetonitrile over five minutes.

For peptides of sub-libraries 1 to 3, 1 μl of the DMSO/DIPEA eluates were diluted into 110 μl of water to give a cyclic peptide concentration of around 120 μM. 5 μl of the samples were injected and analyzed using a 0 to 60% gradient of acetonitrile over five minutes.

For peptides of the peptoid library, 1 or 2.5 μl of the DMSO/DIPEA eluates were diluted with 9 or 7.5 μl acetonitrile to reach a cyclic peptide concentration of around 2 mM. To these dilutions was added 4 μl of TCEP·HCl solution (24 mM in 100 mM MES buffer pH 6.6). 4 μl of the samples were injected and analyzed using a 5 to 80% gradient of acetonitrile over seven minutes.

Analytical HPLC

The purity of peptides was analyzed by reversed-phase analytical HPLC (Agilent Technologies, 1260 Infinity). Peptide released from solid phase were run over a reversed-phase C18 column (Agilent Zorbax 300SB, 300 Å pore, 5 μm particle, 4.6 mm × 250 mm) using a linear gradient of solvent B (acetonitrile, 0.1% TFA) over solvent A (H_2O, 0.1% TFA) from 0−50% in 30 minutes at a flow rate of 1 ml min^{-1}. Typically, 100 nmol of cyclic peptide (around 5 μg) were injected. The mass of the purified cyclic peptides was confirmed by ESI-MS.

Library peptide quantification by absorption

Absorbance measurements were performed with a Nanodrop 8000 spectrophotometer (Thermo Scientific) at a wavelength of 280 nm using a 1 mm path length. Peptides containing Trp were diluted 10-fold and those containing Tyr 5-fold using DMSO so that absorbance measurement values would be in a range from 0.5 to 1.25 units. DMSO with 150 mM DIPEA containing no peptide, diluted 10- and 5-fold with DMSO, showed absorption of 0.023 and 0.039, respectively, showing that the solvent contributed less than 10% to the absorption. The Beer-Lambert law was used to calculate the concentration of the peptides. Extinctions coefficients Trp ε_{280} = 5500 $M^{-1}cm^{-1}$ and Tyr ε_{280} = 1490 $M^{-1}cm^{-1}$ were used. The exact extinction coefficient value for Cl-Trp was not determined and assumed to be ε_{280} = 5500 $M^{-1}cm^{-1}$.

Library peptide quantification by Ellman's reagent

The concentration of peptides was determined by fully reducing the disulfide bonds and quantifying the concentration of free thiol group using the Ellman's assay. The peptides were reduced using TCEP immobilized on beads so that the reducing agent could be removed by filtration before adding Ellman's reagent. A volume of 40 μl of peptide in DMSO, 150 mM DIPEA at an average concentration of around 14 mM (0.56 μmol peptide) was added to 35 mg TCEP beads (2.21 μmol TCEP, around 4 equiv. relative to peptide) in wells of 96-well U-bottom plates (Greiner, 650101) and incubated for two hours under shaking (140 rpm) at room temperature. The solutions were transferred into a 96-well fritted filter plate (Orochem, OF 1100) which was placed on top of a 2 ml deep-well plate (Thermo Scientific, 278752) and centrifuged at 953 g (2000 rpm on a Thermo Heraeus Multifuge 3L-R) for two minutes to collect the reduced peptides.

In a clear 96-well flat-bottom plate (Greiner, 655101) was added 78 μl per well of 3:7 DMSO/60 mM NH_4HCO_3 buffer pH 8 (v/v). 2 μl of the reduced peptide stocks were added to the plate, followed by 20 μl of 20 mM Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid)) in the same DMSO/buffer mixture. The final concentrations of reduced peptide in this assay was in average around 0.28 mM. The final concentration of Ellman's reagent was 4 mM.

Absorbance at 412 nm was measured with a Tecan Infinite M200 Pro plate reader. The absorbance value for Ellman's reagent (4 mM) alone was 0.21 units. The absorbance of peptides, after subtracting 0.21 units ranged from 0.55 to 3.20 units. A standard calibration curve was established by following the above procedure using a peptide with known concentration.

Library screening

Thrombin inhibition by cyclic peptides was assessed by measuring residual activity of thrombin in presence of the cyclic peptides at 11 μM average final concentration. The assays were performed in 384-well plates using Tris buffer at pH 7.4 (100 mM Tris-Cl, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1% w/v BSA, 0.01% v/v Triton-X100, and 1% v/v DMSO) using thrombin at a final concentration of 2 nM and the fluorogenic substrate Z-Gly-Gly-Arg-AMC at a final concentration of 50 μM.

To 384 low-volume assay plates (Nunc, 264705), 12.5 nl cyclic peptide (in DMSO containing 150 mM DIPEA; average cyclic peptide concentration of 13.5 mM) were transferred using acoustic liquid transfer (ECHO 550, Labcyte). Thrombin (7.5 μl, 4 nM) in the Tris buffer described above was added to each peptide using a Gyger Certus Flex liquid dispenser, and incubated for 10 minutes at room temperature. The fluorogenic substrate (7.5 μl, 100 μM) in the same butter was added using the Gyger Certus Flex liquid dispenser, and the florescence intensity measured with a Tecan Infinite M200 Pro fluorescence plate reader (excitation at 360 nm, emission at 465 nm) at 25°C for a period of 30 min with a read every three minutes.

Cyclic peptide purification

A quantity of 50 nmoles of the most active inhibitor was purified on a Thermo Dionex Ultimate 3000 HPLC (Waters Nova-Pak C18 Column, 60 Å pore, 6 μm particle, 7.8 mm × 300 mm) using a 10 to 60% acetonitrile gradient over 20 minutes. The fraction containing the cyclic peptide was dissolved first in 4 μl of DMSO, followed by addition of 196 μl of water.

Determination of the inhibitory constants of thrombin inhibitors

The IC_{50}s of the best cyclic peptide was determined by measuring the residual activity of thrombin at different peptide concentration with the same assay as in the screen. The reactions were performed in volumes of 150 μl in 96-well plates (Greiner, 655101). 50 μl of 2-fold dilutions of cyclic peptide in assay buffer at pH 7.4 (100 mM Tris-Cl, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1% w/v BSA, 0.01% v/v Triton-X100) containing 3% DMSO v/v were pipetted to the wells. A volume of 50 μl of thrombin (6 nM) in assay buffer was added to each well to reach a final concentration of 2 nM and was incubated for 10 minutes. A volume of 50 μl of the fluorogenic substrate Z-Gly-Gly-Arg-AMC (150 μM) in assay buffer containing 1% DMSO v/v was added to reach a final substrate concentration of 50 μM, and 1.33% DMSO. Fluorescence intensity was measured for 30 minutes using a Tecan Infinite M200 Pro plate reader (excitation at 360 nm, emission at 465 nm), with a read every three minutes. Sigmoidal curves were fitted to the data using GraphPad Prism 6 software, and the following dose-response equation:

$$y = 100/1+10(\log IC_{50} - x)p$$

where x is cyclic peptide concentration, y is % protease activity, and p is the Hill slope. IC_{50} values were derived from the fitted curve.

The inhibitory constants (K_i) were calculated using the following equation of Cheng and Prusoff:

$$K_i = IC_{50}/(1 + [S]_0/K_m)$$

where IC_{50} is the functional strength of the inhibitor, $[S]_0$ is the total substrate concentration, and K_m is the Michaelis-Menten constant. The K_m for thrombin and the substrate Z-Gly-Gly-Arg-AMC was determined to be 168 μM.

Supplementary Tables

Supplementary Table 1. Solid phase resins. Properties of five commercial resins used in this work.

	Solid support				
	Aminomethyl ChemMatrix (1)	NovaPEG amino (2)	Hypogel 200 NH_2 (3)	Aminomethyl polystyrene (4)	Polystyrene A SH (5)
Provider	Sigma	Novabiochem	Sigma	Aapptec	Rapp Polymere
Polymer	PEG	PEG	PEGylated polystyrene	Polystyrene	Polystyrene
Particle size	35-100 mesh	35-100 mesh	100-140 mesh	100-200 mesh	200-400 mesh
Capacity	1 mmol g^{-1}	0.53 mmol g^{-1}	0.90 mmol g^{-1}	1.39 mmol g^{-1}	0.95 mmol g^{-1}
Property	polar	polar	polar	apolar	apolar
Functional group	NH_2	NH_2	NH_2	NH_2	SH

Supplementary Table 2. Synthesis of peptides immobilized via a disulfide bridge. Values are means of two independent syntheses and cleavage reactions.

	Solid support				
	Aminomethyl ChemMatrix (1)	NovaPEG amino (2)	Hypogel 200 NH_2 (3)	Aminomethyl polystyrene (4)	Polystyrene A SH (5)
Scale (μmol)	100	100	200	100	100
Syringe tare (mg)	2234.6	2244.5	2232.5	2251.1	2236.5
Initial resin weight (mg)	101.6	189.0	225.5	72.4	105.9
Resin weight after S-S exchange (mg)	107.6	205	236.3	79.2	109.5
Resin weight after SPPS (mg)	131.1	233.6	270.2	119.6	195.4
Amount of peptide (protected) on resin (mg)	23.5	28.6	33.9	40.4	85.9
Amount of resin transferred to 96-well plate for 5 μmoles (mg)	6.6	11.7	6.8	6.0	9.8

Supplementary Table 3. Reductive cleavage in 9:1 v/v DMSO/water. Concentrations and % recovery are indicated. Values are means of two independent syntheses and cleavage reactions.

Reducing agent	Performance	Solid support				
		Aminomethyl ChemMatrix (1)	NovaPEG amino (2)	Hypogel 200 NH_2 (3)	Aminomethyl polystyrene (4)	Polystyrene A SH (5)
TCEP	Concentration (μM)	300	320	2100	6000	7300
	% recovery	1.2	1.3	8.5	24	29
Solvent only	Concentration (μM)	118	109	13	350	98
	% recovery	0.47	0.44	0.05	1.4	0.39

Supplementary Table 4. Reductive cleavage in 17:3 v/v MeCN/water. Concentrations and % recovery are indicated. Values are means of two independent syntheses and cleavage reactions.

Reducing agent	Performance	Solid support				
		Aminomethyl ChemMatrix (1)	NovaPEG amino (2)	Hypogel 200 NH_2 (3)	Aminomethyl polystyrene (4)	Polystyrene A SH (5)
TCEP	Concentration (μM)	830	970	3700	3500	5100
	% recovery	3.3	3.9	15	14	21
solvent only	Concentration (μM)	260	470	18	760	330
	% recovery	1	1.9	0.1	3.1	1.3

Supplementary Table 5. Yields and purity of cyclic peptide Mpa-Gly-Gln-Trp-Mea obtained with the cyclative release strategy. The peptide Mpa-Gly-Gln-Trp-Mea was synthesized on five resins at a 5 μmol scale, followed by on-resin removal of side-chain protecting groups. Cleavage was performed with either 200 μl of 150 mM DIPEA in DMSO, or 200 μl of 1:9 buffer/DMSO (v/v) overnight (buffer = 150 mM NH_4HCO_3 pH 8.0). All steps were performed in triplicate. Percent recovery was determined by measuring absorbance at 280 nm. Purity was determined by measuring the area under the curve of UHPLC chromatograms recorded at 220 nm. The detection limit of the UHPLC was estimated to be around 0.5 % recovery. N.D. = not determined.

Cyclative release condition	Performance	Solid support				
		Aminomethyl ChemMatrix (1)	NovaPEG amino (2)	Hypogel 200 NH_2 (3)	Aminomethyl polystyrene (4)	Polystyrene A SH (5)
150 mM DIPEA in DMSO	% Recovery	< 0.5	< 0.5	< 0.5	21 ± 5	44 ± 4
	% Purity of cleaved cyclic peptides	N. D.	N. D.	N. D.	95 ± 4	93 ± 1
150 mM DIPEA in 1:9 buffer:DMSO	% Recovery	< 0.5	< 0.5	< 0.5	N. D.	N. D.
	% Purity of cleaved cyclic peptides	N. D.	N. D.	N. D.	N. D.	N. D.

Supplementary Table 6. Quantification of cyclic peptides and side products. The relative abundance of all species was determined based on the area under the curve of the analytical HPLC spectra shown in Figure 2b.

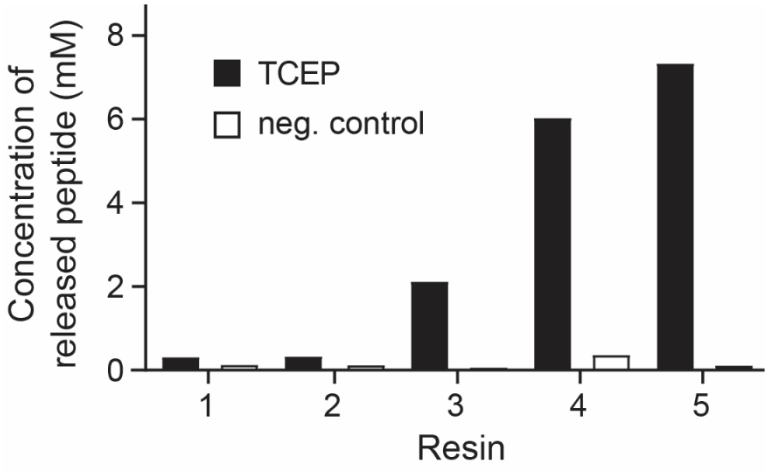
Cyclic peptide	Recovery[a]	Product	Side products			
		Cyclic peptide	Linear	Dimer	tBu	Other
1	67%	86%	6%	4%	4%	0%
2	94%	96%	0%	0%	0%	4%[b]
3	108%	90%	0%	10%	0%	0%
4	54%	75%	0%	17%	0%	8%[c]

[a]Relative to yield expected based on resin loading. [b]Side products M/Z = Product +53 or +133 Da. [c]Side product M/Z = product + 100 Da.

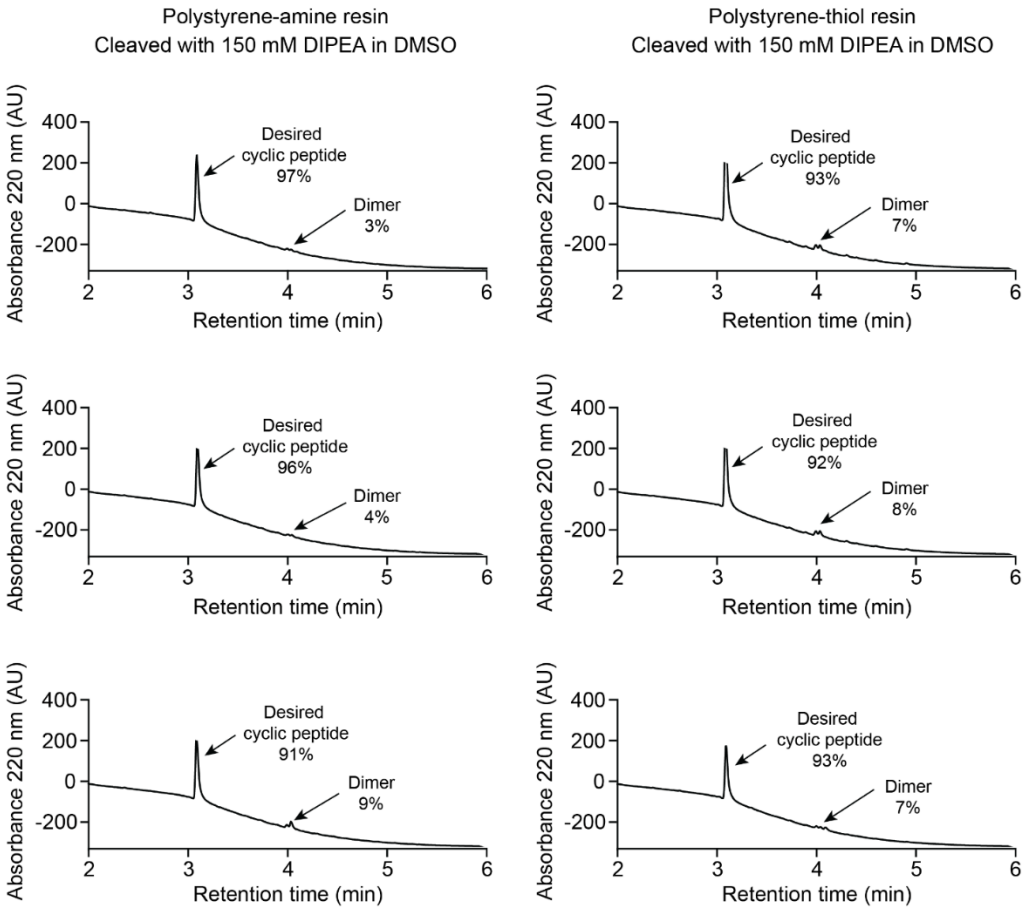
Supplementary Table 7. Quantification of cyclic peptides and side products. The relative abundance of all species was determined based on the area under the curve of the analytical HPLC spectra shown in Supplementary Figure 3b. [a]Relative to yield expected based on resin loading. [b]Side product M/Z = Product + 53 Da. [c]Side product M/Z = Product + 133 Da.

		Products				
Amino acid (cyclic peptide)	Recovery[a]	Cyclic peptide	Linear	Dimer	tBu	Other
Trp (5)	67%	68%	6%	18%	8%	0%
3-AMBA (6)	71%	68%	0%	15%	0%	17%[b]
Pro (7)	79%	49%	7%	37%	0%	7%[c]
Nipecotic (8)	31%	82%	0%	18%	0%	0%

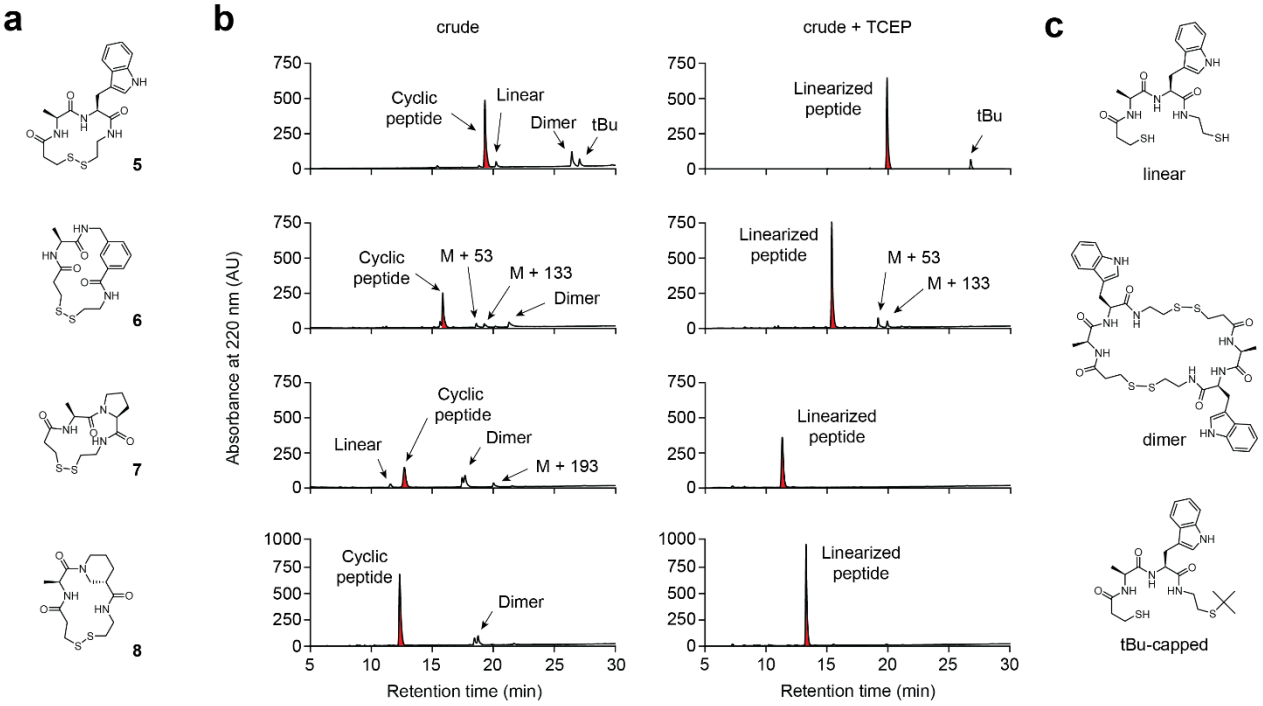
Supplementary Figures



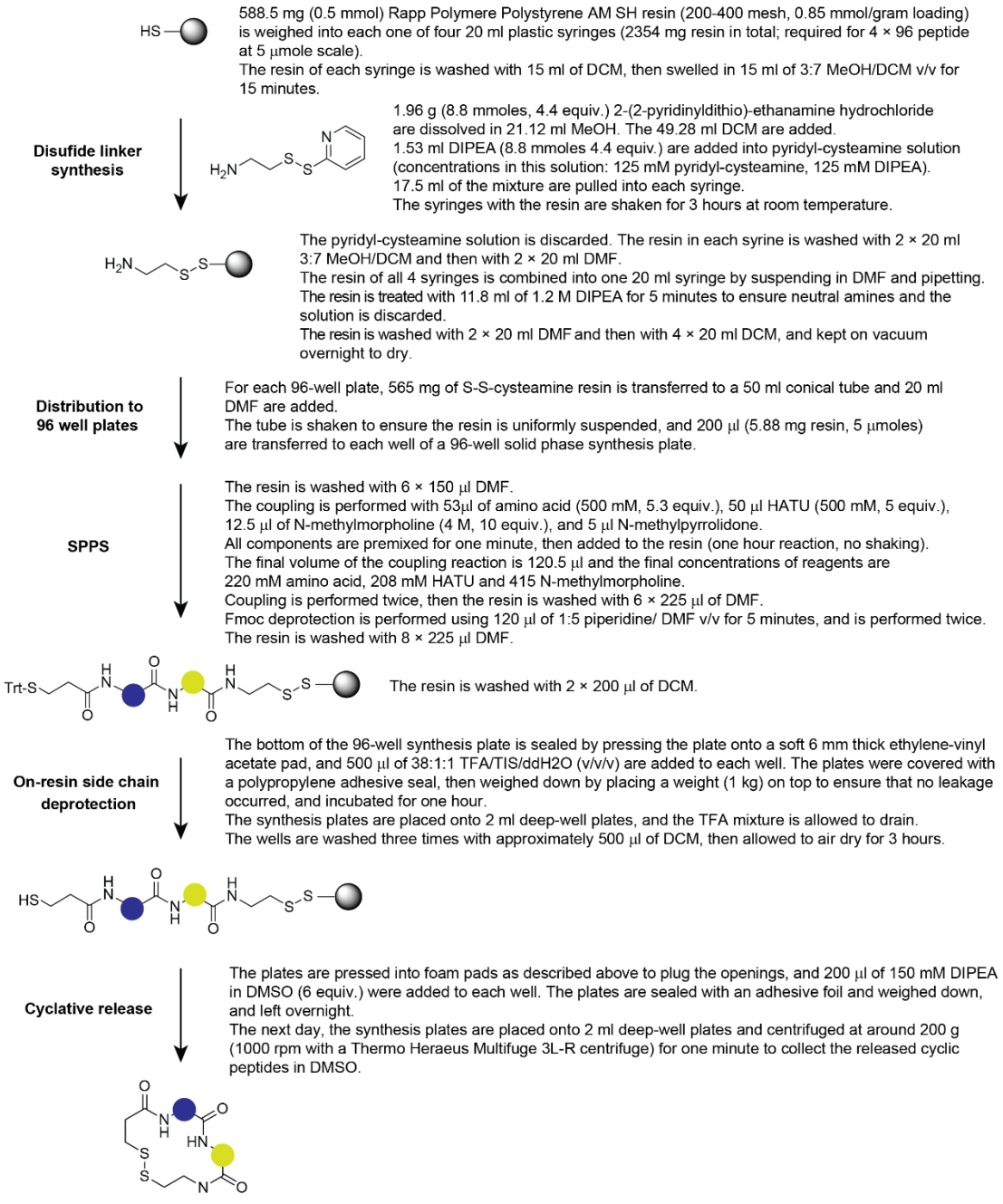
Supplementary Figure 1. Release of peptide by disulfide bridge reduction with TCEP. Yields of Mpa-Gly-Gln-Trp-Mea upon incubation of the five resins (5 μmol scale) with the reducing agent TCEP in 9:1 DMSO/water (v/v), wherein the negative control represents solvent only. Yields are indicated according to the concentration in the 200 μl eluates, determined by measuring absorbance at 280 nm. Reactions were perfomed in duplicate, and the average recovery values are shown.



Supplementary Figure 2. LC-MS chromatograms of model peptide Mpa-Gly-Gln-Trp-Mea obtained by cyclative release. The peptide was synthesized on resin 4 (Aminomethyl polystyrene resin) or resin 5 (Polystyrene A SH resin). Following SPPS and on-resin removal of side-chain protecting groups, the peptides were released by treatment with 150 mM DIPEA in DMSO. Quantities of product and side-products were determined by measuring the area under the curve of peaks in the UHPLC chromatograms at 220 nm absorbance. Synthesis and cleavage were performed in triplicate for each resin.



Supplementary Figure 3. Cyclative release of peptides containing four building blocks. (a) Chemical structures of the desired four cyclic peptides. The peptides are based on the linear sequence Mpa-Ala-Xaa-Mea with “Xaa” being an amino acid with variable conformational flexibility in the backbone. (b) Analytical HPLC chromatograms of the crude peptides after cyclative release. The chromatograms on the right show the peptides after disulfide-bond reduction with TCEP. For impurities that were not identified, the mass is indicated. (c) Examples of chemical structures that fit with the molecular masses of the identified impurities.



Supplementary Figure 4. One-page recipe for the synthesis and cyclative release of peptides in four 96-well plates (384 peptides in total). Note: the protocol is slightly different than procedures described in the materials and methods section for model peptides, as the resin was prepared in batch at a larger scale, and as some procedures were slightly optimized.

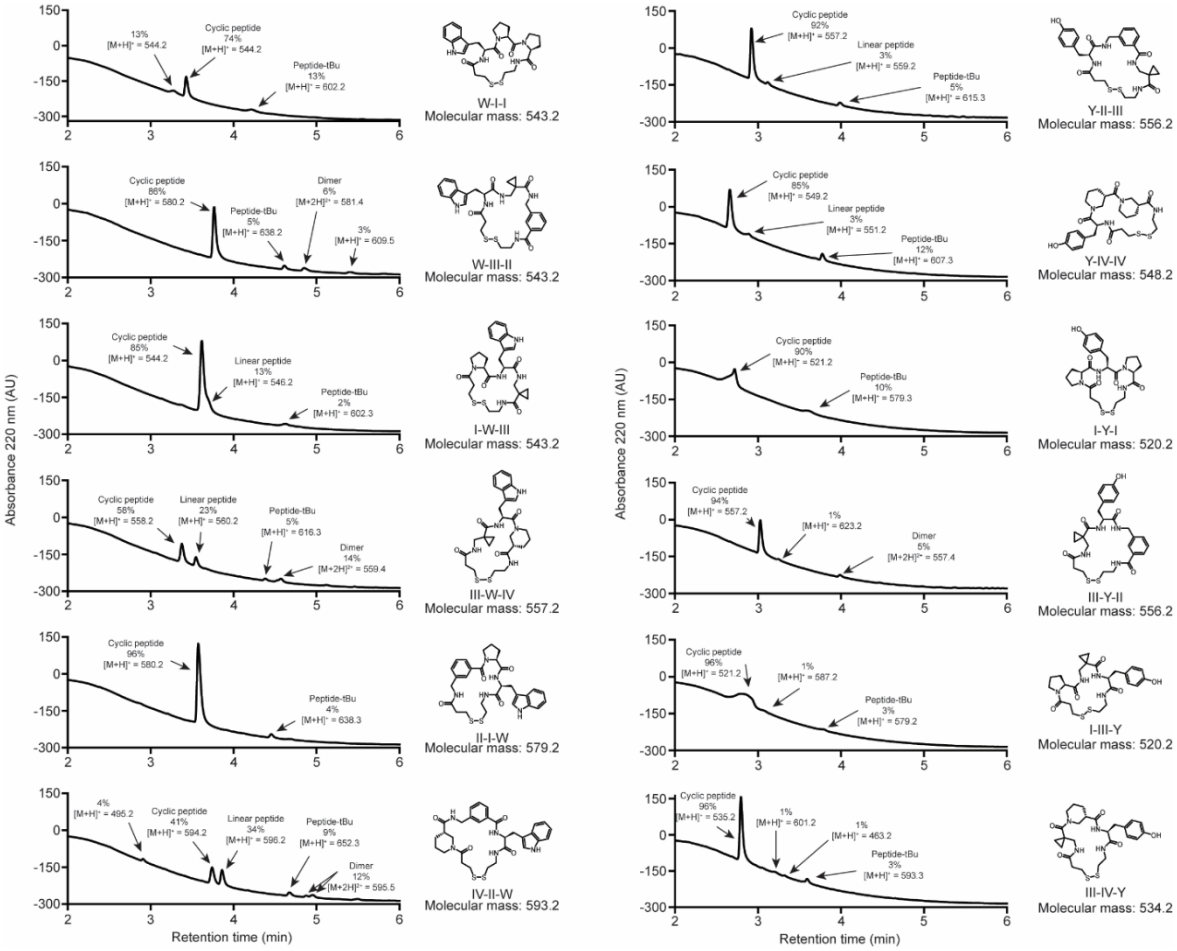
a

	1	2	3	4	5	6	7	8	9	10	11	12
A	W-I-I	W-III-I	I-W-I	III-W-I	I-I-W	III-I-W	Y-I-I	Y-III-I	I-Y-I	III-Y-I	I-I-Y	III-I-Y
B	W-I-II	W-III-II	I-W-II	III-W-II	I-II-W	III-II-W	Y-I-II	Y-III-II	I-Y-II	III-Y-II	I-II-Y	III-II-Y
C	W-I-III	W-III-III	I-W-III	III-W-III	I-III-W	III-III-W	Y-I-III	Y-III-III	I-Y-III	III-Y-III	I-III-Y	III-III-Y
D	W-I-IV	W-III-IV	I-W-IV	III-W-IV	I-IV-W	III-IV-W	Y-I-IV	Y-III-IV	I-Y-IV	III-Y-IV	I-IV-Y	III-IV-Y
E	W-II-I	W-IV-I	II-W-I	IV-W-I	II-I-W	IV-I-W	Y-II-I	Y-IV-I	II-Y-I	IV-Y-I	II-I-Y	IV-I-Y
F	W-II-II	W-IV-II	II-W-II	IV-W-II	II-II-W	IV-II-W	Y-II-II	Y-IV-II	II-Y-II	IV-Y-II	II-II-Y	IV-II-Y
G	W-II-III	W-IV-III	II-W-III	IV-W-III	II-III-W	IV-III-W	Y-II-III	Y-IV-III	II-Y-III	IV-Y-III	II-III-Y	IV-III-Y
H	W-II-IV	W-IV-IV	II-W-IV	IV-W-IV	II-IV-W	IV-IV-W	Y-II-IV	Y-IV-IV	II-Y-IV	IV-Y-IV	II-IV-Y	IV-IV-Y

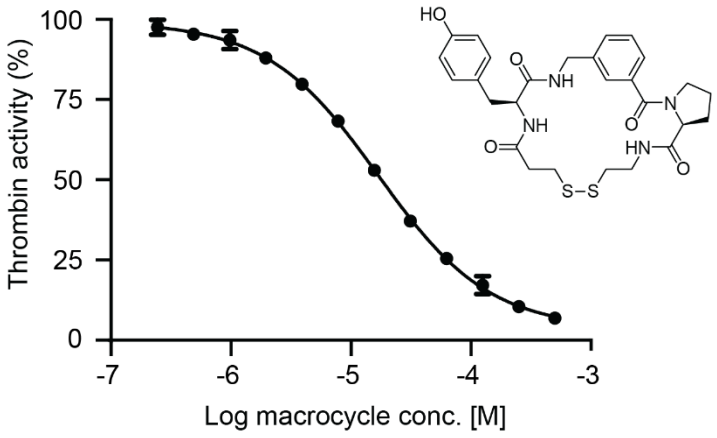
b

Well	Sequence	% Product	% Linear	% tBu	% Dimer	% other
A1	Mpa-W-I-I-Mea	87	0	13	0	0
B2	Mpa-W-III-II-Mea	86	0	5	6	3
C3	Mpa-I-W-III-Mea	85	13	2	0	0
D4	Mpa-III-W-IV-Mea	58	23	5	14	0
E5	Mpa-II-I-W-Mea	96	0	4	0	0
F6	Mpa-IV-II-W-Mea	41	34	9	12	4
G7	Mpa-Y-II-III-Mea	92	3	5	0	0
H8	Mpa-Y-IV-IV-Mea	85	3	12	0	0
A9	Mpa-I-Y-I-Mea	90	0	10	0	0
B10	Mpa-III-Y-II-Mea	94	0	0	5	1
C11	Mpa-I-III-Y-Mea	96	0	3	0	1
D12	Mpa-III-IV-Y-Mea	92	0	6	0	2

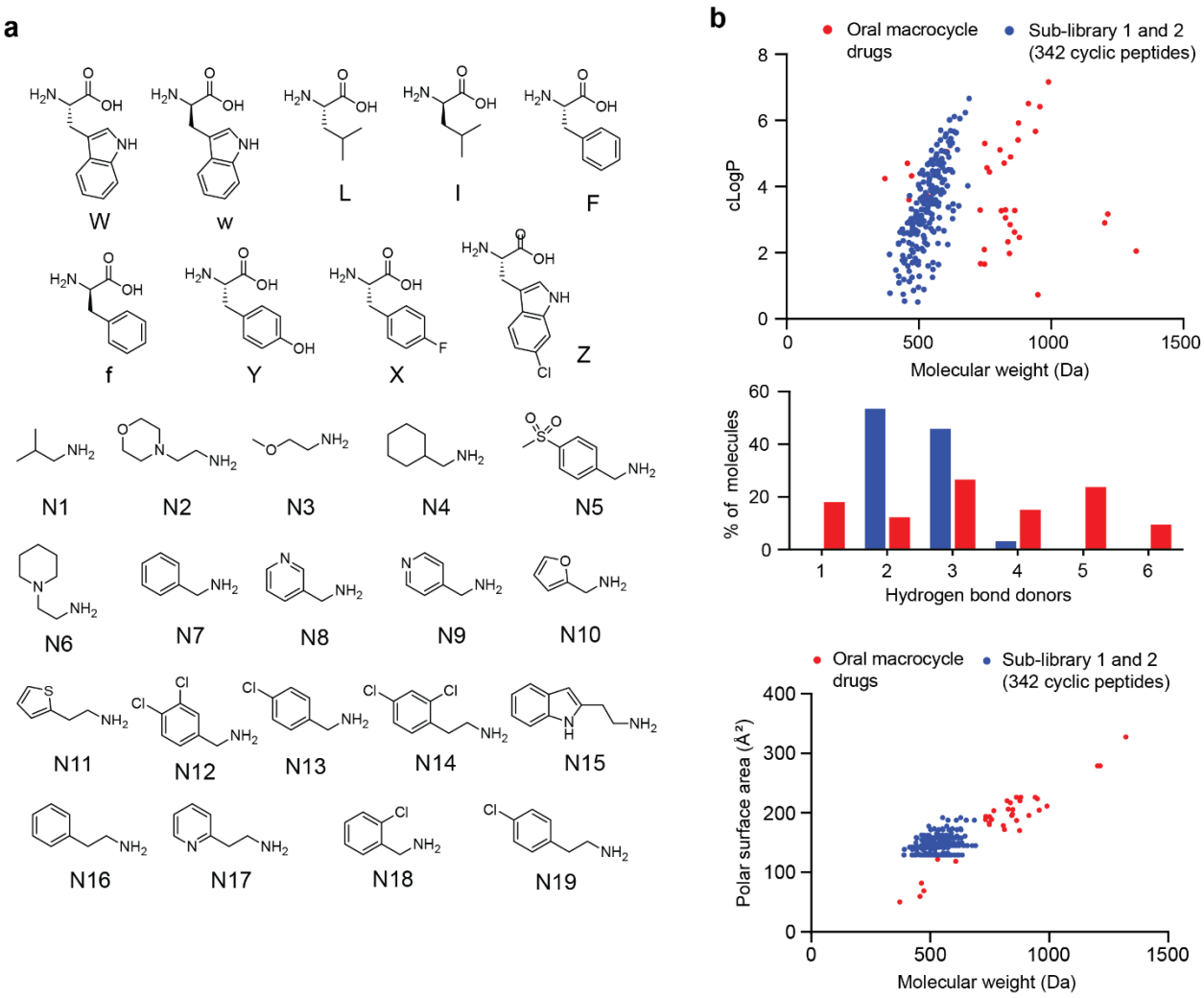
Supplementary Figure 5. Sample peptides analyzed from the 96 cyclic peptide library. (a) Layout of the synthesis plate showing the sample peptides that were randomly selected (yellow). (b) Purity of peptides analyzed by LC-MS analysis. The area under the curve of peaks recorded at 220 nm was determined and the % of each species calculated. The codes for the amino acid building blocks shown in Figure 3 are indicated.



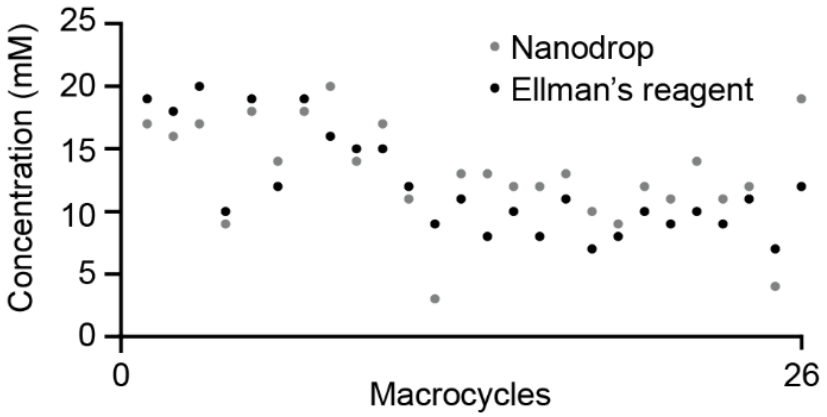
Supplementary Figure 6. UHPLC chromatograms of the 12 sample peptides analyzed from the 96 cyclic peptide sub-libraries 1 to 3. Samples were run on a 0 to 60% MeCN gradient over six minutes. For impurities that were not identified, only the observed mass is indicated. The chemical structures and molecular masses (monoisotopic masses) of the desired cyclic peptides are indicated.



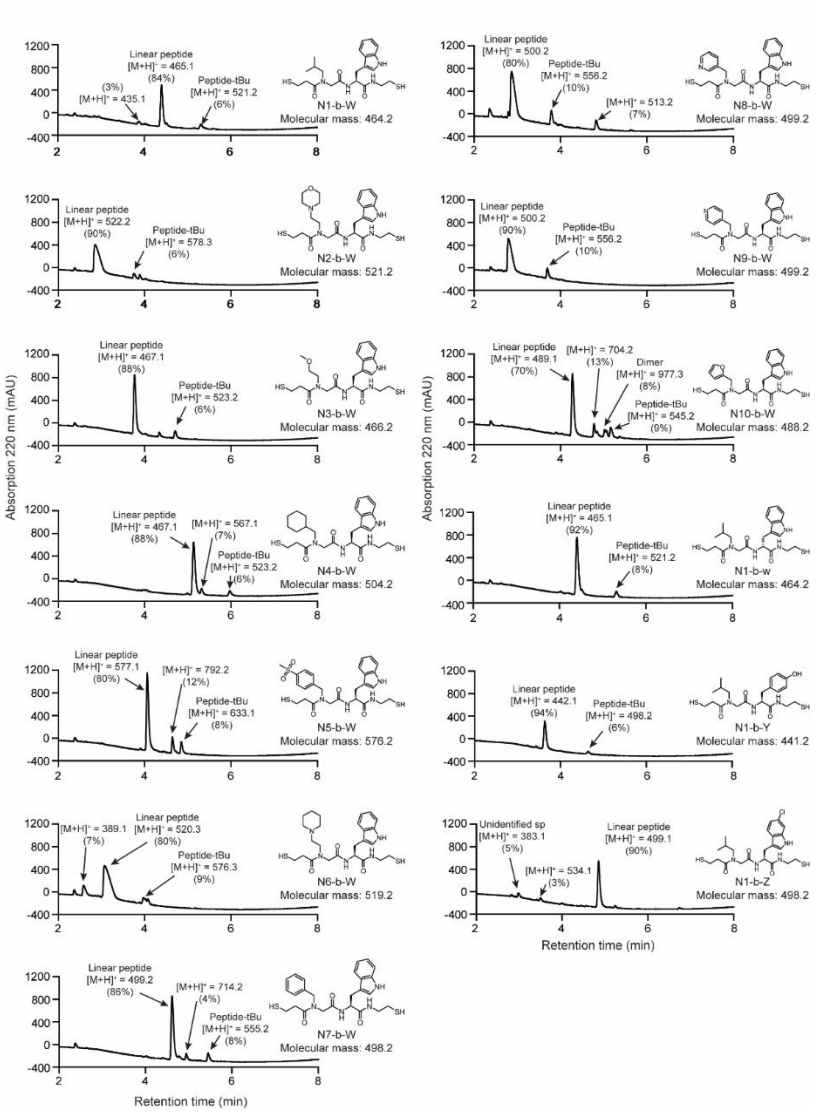
Supplementary Figure 7. Activity of the most active hit found in the thrombin screen. Residual activity of thrombin is shown for increasing concentrations of the HPLC-purified cyclic peptide Mpa-Tyr-Il-Mea. Mean values and standard deviations of three independent measurements are shown.



Supplementary Figure 8. Library composed of 342 small cyclic N-alkylated peptides. (a) Building blocks used for the synthesis of the combinatorial library. (b) Comparison of molecular weight, calculated logP, number of H-bond donors, and polar surface area in cyclic peptides of the library and 34 oral macrocycle drugs or drug candidates. Values were calculated using DataWarrior. Oral macrocycle drugs were taken from Kihlberg and colleagues.

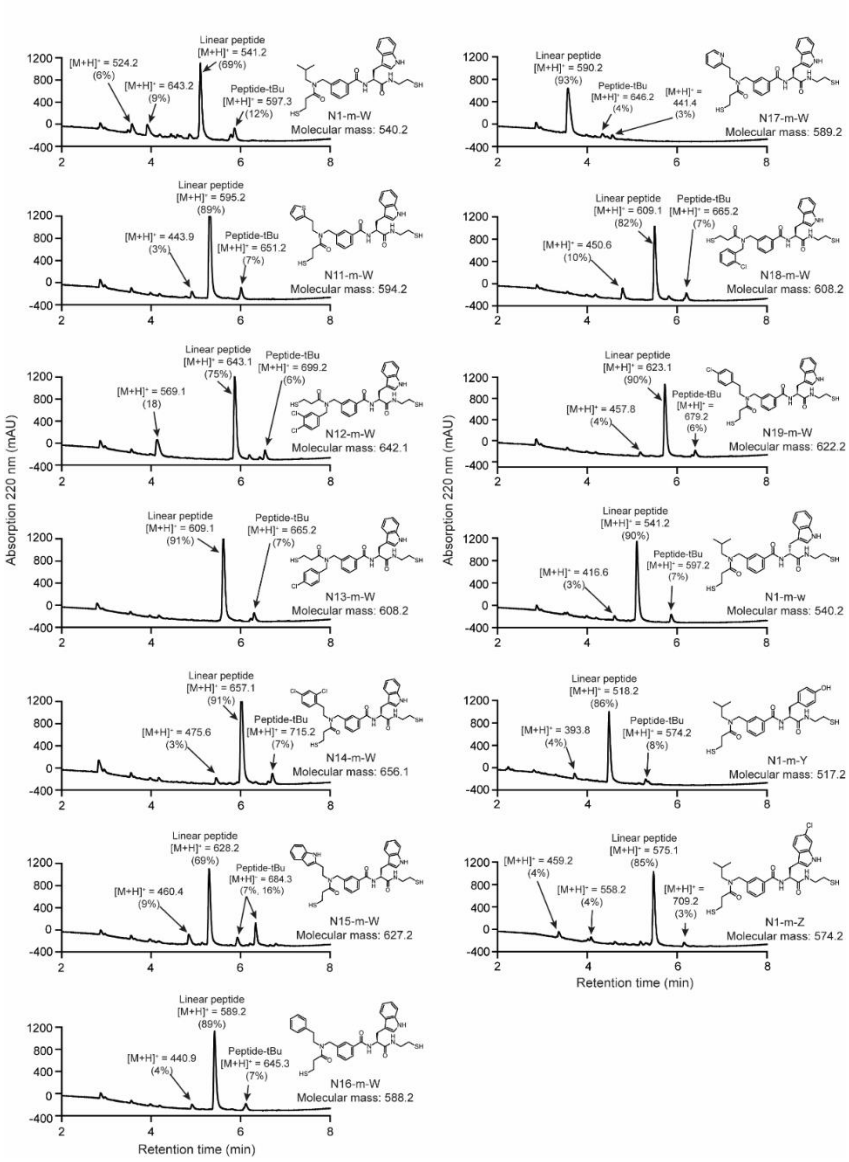


Supplementary Figure 9. Quantification of cyclic peptides based on absorbance and number of thiol groups.



Supplementary Figure 10. LC-MS analysis of the peptide/peptoid library. 26 samples of the 342-member library, reduced for concentration determination using Ellman's reagent, were analyzed by LC-MS. Sample peptides were chosen as follows: half of the peptides are from sub-library 1 and 2, respectively. From sub-library 1, peptides containing L-Trp and the amine building blocks N1-N9 were analyzed, as well as three peptides containing D-Trp, L-Tyr, and L-Cl-Trp. From sub-library 2, peptides containing L-Trp and the amine building blocks N1 and N10-N19 were analyzed, as well as three peptides containing D-Trp, L-Tyr, and L-Cl-Trp. The percentage of desired peptide was determined by quantifying the area under the curve of product and side-

product peaks recorded at 220 nm. Samples were run on a 5-80% MeCN gradient over six minutes.



Supplementary Figure 10. Continued.