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Supporting Information for

A releasable disulfide-linked peptide tag facilitates the synthesis and purification of short peptides

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Supplementary Results

Cyclization of model peptide Y-R in presence of tag

We tested if the released peptide Y-R, in presence of the released tag and the reducing agent TCEP, can be cyclized with the bis-electrophilic linker reagents **1**-**5** shown in Figure 2a. We incubated Y-R-Tag (6.7 mM) with two equiv. TCEP (13 mM) in DMSO to release the Y-R peptide, added the products to the reagents **1**-**5** dissolved in ammonium carbonate buffer and DMSO, and incubated the reactions for 1 hr at room temperature. The final concentrations and solvent fractions were 0.33 mM Y-R-Tag, 0.67 mM TCEP, 2 mM reagent **1**-**5**, 15% DMSO, and 85% 60 mM NH_4HCO_3 buffer, pH 8. LC-MS analysis showed that the Y-R peptide was efficiently released and cyclized by all linkers with yields above 50% (Fig. 2c). Chromatograms of the reactions are shown in Figure 2d for the cyclization with reagent **1** and in Figure S1 for the other reagents. To identify more easily the reaction products and side products, we performed control reactions in which Y-R peptide only (purified), Tag only (purified) and no peptide were incubated with the bis-electrophile reagents (Fig. 2d and Fig. S1).

Supplementary Materials and Methods

Solid phase synthesis of Boc-Cys(Npys)-Gly-Arg-Trp

Resin-bound Npys-Tag was synthesized on an automated peptide synthesizer (Advanced ChemTech 348Ω, AAPPTec) by standard Fmoc (fluorenylmethyloxycarbonyl) solid-phase chemistry on Rink Amide PEGA resin (0.32 mmol/g, 30 μmol scale), using *N*,*N*´-dimethylformamide (DMF) as a solvent (99.5% pure). Couplings were performed twice for each amino acid (4 equiv.) using hexafluorophosphate azabenzotriazole tetramethyl uronium (HATU; 4 equiv.) and N,N-Diisopropylethylamine (DIPEA; 6 equiv.) in 1.45 mL of DMF with shaking at 400 rpm for 60 min. The resin was washed four times with 3 mL of DMF. Fmoc groups were removed by incubation twice with 2.5 mL of 20% (v/v) piperidine in DMF with shaking at 400 rpm for 5 min. The resin was then washed four times with 3 mL of DMF. The deprotection step with piperidine was not performed after the coupling of Boc-Cys(Npys)-OH. At the end of the synthesis, the resin was washed twice with 3 mL of DMF and stored in DMF. In all washing steps, the resin was shaken at 400 rpm for 1 min.

Disulfide exchange reaction on resin

Resin-bound Npys-Tag (30 μmol) was washed trice with water (4 mL) and then treated with a solution of cysteamine (80 mM, 8 equiv.) in acetic acid (3 mL, 1 M, pH 2). The mixture was rotated for 50 min, before the resin was washed four times with water (4 mL) and then four times with DMF (4 mL) to give resin-bound Cysteamine-Tag.

Solid phase synthesis of disulfide-linked peptide-tag (Y-R-Tag model peptide)

Y-R-Tag model peptide was synthesized on an automated peptide synthesizer (Intavis, MultiPep RSi) by standard Fmoc solid-phase chemistry on resin-bound Cysteamine-Tag (0.32 mmol/g, 50 μmol scale), using DMF as a solvent (99.5% pure). Couplings were performed twice for each amino acid (4 equiv.) using HATU (4 equiv.) and N-methylmorpholine (NMM; 10 equiv.) in 0.9 mL of DMF with shaking at 400 rpm for 45 min. The resin was washed seven times with 2 mL of DMF. Fmoc groups were removed by incubation twice with 0.8 mL of 20% (v/v) piperidine in DMF with shaking at 400 rpm for 5 min. The resin was then washed seven times with 2 mL of DMF. At the end of the synthesis, the resin was washed twice with 0.6 mL of dichloromethane (DCM). In all washing steps, the resin was shaken at 400 rpm for 1 min.

Cleavage of Y-R-Tag model peptide from resin

The Y-R-Tag model peptide was cleaved from the resin and the protecting groups removed with 5 mL of cleavage solution (95% trifluoroacetic acid, 2.5% triisopropylsilane, 2.5% water) for 2 hours under shaking at room temperature (RT). The cleavage solution was filtered and then evaporated under a stream of nitrogen. The residue was dissolved in 10 mL of water/acetonitrile (9:1) containing 0.1% TFA before purification with a high-performance liquid chromatography (HPLC) system (Prep LC 2535 HPLC, Waters).

HPLC purification of Y-R-Tag model peptide

Y-R-Tag model peptide was purified by HPLC using a reversed phase preparative C18 column (Waters Sunfire™, 10 μm, 100 Å, 19 × 250 mm) applying a flow rate of 20 ml/min and a linear gradient of 5 to 30% (v/v) solvent B for 30 min [solvent A: 99.9% (v/v) water and 0.1% (v/v) trifluoroacetic acid; solvent B: 99.9% (v/v) acetonitrile and 0.1% (v/v) trifluoroacetic acid]. Fractions containing the desired peptide were lyophilized.

TCEP mediated release of tag

To a solution of Y-R-Tag (3 μL, 20 mM) in DMSO was added TCEP (6 μL, 20 mM, 2 equiv.) in DMSO. The mixture was incubated at RT for 30 min. Complete disulfide reduction afforded peptide Y-R (6.7 mM) and Tag (6.7 mM). A Shimadzu-2020 single quadrupole LC-MS system was used for sample analysis. The disulfide reduction mixture (9 μL) was diluted 10-fold by addition of water/acetonitrile (7:3) (81 μL). Samples were analyzed using a revered phase C18 column (injection volume of 1 μL, Phenomenex Kinetex®, 2.6 μm, 100 Å, 50 × 2.1 mm) using a linear gradient of solvent B (acetonitrile, 0.05% formic acid) over solvent A (water, 0.05% formic acid) from 5 to 30% in 5 min at a flow rate of 1 ml/min, and by mass analysis in positive mode.

Macrocyclization of released Y-R peptide with bis-electrophile reagents

Cyclization reagent (2 μL, 20 mM, 6 equiv.) in DMSO was added to reaction buffer (17 μL, 60 mM ammonium bicarbonate, pH 8). TCEP-reduced Y-R-Tag (1 μL, 6.7 mM Y-R, 6.7 mM Tag, 13.3 mM TCEP) in DMSO was then added, and the reaction mixture was incubated for 1 hour at RT.

Control 1: Cyclization reagent (2 μL, 20 mM, 6 equiv.) in DMSO was added to reaction buffer (17 μL, 60 mM ammonium bicarbonate, pH 8). Purified Y-R (1 μL, 6.7 mM) in DMSO was then added, and the reaction mixture was incubated for 1 hour at RT.

Control 2: Cyclization reagent (2 μL, 20 mM, 6 equiv.) in DMSO was added to reaction buffer (17 μL, 60 mM ammonium bicarbonate, pH 8). Purified Tag (1 μL, 6.7 mM) in DMSO was then added, and the reaction mixture was incubated for 1 hour at RT.

Control 3: Cyclization reagent (2 μL, 20 mM, 6 equiv.) in DMSO was added to reaction buffer (17 μL, 60 mM ammonium bicarbonate, pH 8). TCEP (1 μL, 13.3 mM) in DMSO was then added, and the reaction mixture was incubated for 1 hour at RT.

LC-MS analysis of macrocyclization reactions

A Shimadzu-2020 single quadrupole LC-MS system was used for sample analysis. The macrocyclization reaction mixtures (20 μL) were diluted 10-fold by addition of water/acetonitrile (7:3) (180 μL). Samples were analyzed using a revered phase C18 column (injection volume of 10 μL, Phenomenex Kinetex®, 2.6 μm, 100 Å, 50×2.1 mm) applying a linear gradient of solvent B (acetonitrile, 0.05% formic acid) over solvent A (water, 0.05% formic acid) from 0 to 40% in 5 min at a flow rate of 1 mL/min, and by mass analysis in positive mode.

Solid phase synthesis of disulfide-tagged peptide library

Library peptides were appended to resin-bound Cysteamine-Tag (0.32 mmol/g, 5 μmol scale) by standard Fmoc solid-phase chemistry in a 96-well peptide synthesis filter plate (32.410, Intavis) using an automated peptide synthesizer (Intavis MultiPep RSi), and DMF as a solvent (99.5% pure). Couplings were performed twice for each amino acid (4 equiv.) using HATU (4 equiv.) and NMM (10 equiv.) in 130 μL of DMF without shaking for 45 min. The resin was washed six times with 225 μL of DMF. Fmoc groups were removed by incubation twice with 120 μL of 20% (v/v) piperidine in DMF without shaking for 5 min. The resin was then washed eight times with 200 μL of DMF. At the end of the synthesis, the resin was washed twice with 200 μL of DCM. In all washing steps, the resin was incubated for 1 min. The peptides were deprotected and cleaved from the resin in the 96-well peptide synthesis filter plate with cleavage solution (95% trifluoroacetic acid, 2.5% triisopropylsilane, 2.5% water). Volumes of 300 μL, 100 μL, and again 100 μL were added sequentially to the wells of the reaction plate and incubated for 30 min at RT after each addition. The flow-through was collected in a 96

deep well plate (32.296, Intavis), and the volume of cleavage solution was reduced to approximately 100 μL under a stream of nitrogen. Peptides were precipitated by the addition of cold diethyl ether (1.2 mL) and incubated at -20 °C for 16 hours. After centrifugation at 4,000 g for 30 min, the peptide pellets were dissolved in acetonitrile/water (1:1) and lyophilized. The lyophilized residue was first dissolved in DMSO (10 μL), and then 1 μL of each DMSO stock was further diluted 100-fold in water for concentration measurement, concentration adjustment and LC-MS analysis. Concentrations were determined by measuring absorption at 280 nm using a nanodrop 8000 (ThermoScientific). The concentration was standardized to 20 mM by the addition of DMSO. Peptides with lower concentrations were not concentrated for further experiments. A Shimadzu-2020 single quadrupole LC-MS system was used for sample analysis. Samples were analyzed using a reversed phase C18 column (injection volume of 4 μL, Phenomenex Kinetex®, 2.6 μm, 100 Å, 50 × 2.1 mm) using a linear gradient of solvent B (acetonitrile, 0.05% formic acid) over solvent A (water, 0.05% formic acid) from 0 to 40% in 5 min at a flow rate of 1 ml/min, and by mass analysis in positive mode.

Production of HPLC-purified cyclic peptides 1 and 2

The linear precursors of the cyclic peptides 1 and 2 were synthesized on an automated peptide synthesizer (Intavis, MultiPep RSi) by standard Fmoc solid-phase chemistry on cysteamine 4-methoxytrityl resin (0.92 mmol/g, 50 μmol scale), using DMF as a solvent (99.5% pure). Couplings were performed twice for each amino acid (4 equiv.) using HATU (4 equiv.) and NMM (10 equiv.) as described above. Peptides were cleaved by incubation with 5 mL of cleavage solution (95% trifluoroacetic acid, 2.5% triisopropylsilane, 2.5% water) for 2 hours under shaking at RT. The cleavage solution was filtered and then evaporated under a stream of nitrogen. The residue was dissolved in 10 mL of water/acetonitrile (9:1) containing 0.1% TFA and purified by HPLC as described previously for the Y-R-Tag model peptide.

Linear peptides (1 equiv.) were cyclized in reaction buffer (60 mM ammonium bicarbonate, pH 8) by the addition of reagent **1** (4 equiv.) in acetonitrile. The reaction mixture was incubated for 1 hour at RT, lyophilized and purified by HPLC. Both linear and cyclized peptides were purified using a reversed phase preparative C18 column (Waters Sunfire™, 10 μm, 100 Å, 19 × 250 mm) applying a flow rate of 20 ml/min and a linear gradient of 5 to 30% (v/v) solvent B for 30 min [solvent A: 99.9% (v/v) water and 0.1% (v/v) trifluoroacetic acid; solvent B: 99.9% (v/v) acetonitrile and 0.1% (v/v) trifluoroacetic acid]. Fractions containing the desired linear/cyclic peptides were lyophilized.

Production of cyclic peptides 1 and 2 using the tag-based strategy

The linear precursors of the cyclic peptides 1 and 2 were synthesized as conjugates with the tag in a 96-well peptide synthesis filter plate, cleaved from the resin together with the tag, precipitated by diethyl ether, adjusted to 20 mM concentration in DMSO, released from the tag by TCEP-reduction, and cyclized by reagent **1**, all following the protocols described above. The peptides and side products were analyzed on a Shimadzu-2020 single quadrupole LC-MS system as described above.

Thrombin inhibition assays

The IC_{50}s of HPLC-purified cyclic peptides 1 and 2 were determined by measuring the residual activity of thrombin at different dilutions of macrocycle using a fluorogenic substrate. The reactions were performed in a volume of 150 μL in 96-well plates (Greiner Bio-One, 96 well, Clear, Flat Bottom, Polystyrene Microplate). A volume of 50 μL of 2-fold dilutions of macrocycle in assay buffer (10 mM Tris-Cl, pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1% w/v BSA, 0.01% v/v Triton-X100) was 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 15 min. A volume of 50 μL of the fluorogenic substrate Z-Gly-Gly-Arg-AMC (150 μM) in assay buffer containing 3% DMSO was added to reach a final substrate concentration of 50 μM and 1% DMSO. In the negative control, assay buffer was used in place of peptide. Experiments were conducted in triplicate.

Fluorescence intensity was measured at 25 °C for 30 min using a Tecan Infinite M200Pro plate reader (excitation at 368 nm, emission at 467 nm) with a read every minute. Sigmoidal curves were fitted to the data using Prism 5 (GraphPad software) and the following dose-response equation:

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

wherein x is macrocycle concentration, y is % protease activity, and p is 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:

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

wherein 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.

The thrombin inhibition assays with cyclic peptides 1 and 2 produced with the tag-based strategy were performed in a volume of 20 μL in a 384-well plate (384 well, black, flat bottom, shallow well, Nunc™ Microplate). A volume of 2 μL of undiluted crude reaction mixture was pipetted to the wells. A volume of 13 μL of thrombin (3 nM) in assay buffer was added to each well to reach a final concentration of 2 nM and incubated for 15 min. A volume of 5 μL of substrate Z-Gly-Gly-Arg-AMC (200 μM) in assay buffer containing 4% DMSO was added to reach a final substrate concentration of 50 μM and 1% DMSO. The residual protease activity was measured at 25 °C by monitoring the change of fluorescence intensity (excitation at 368 nm, emission at 467 nm) over 30 min using a Tecan Infinite F500 microplate reader with readings taken at 3 min intervals. The extent of inhibition (%) was calculated by comparing the residual activity to a negative control without peptide.

Supplementary Figures

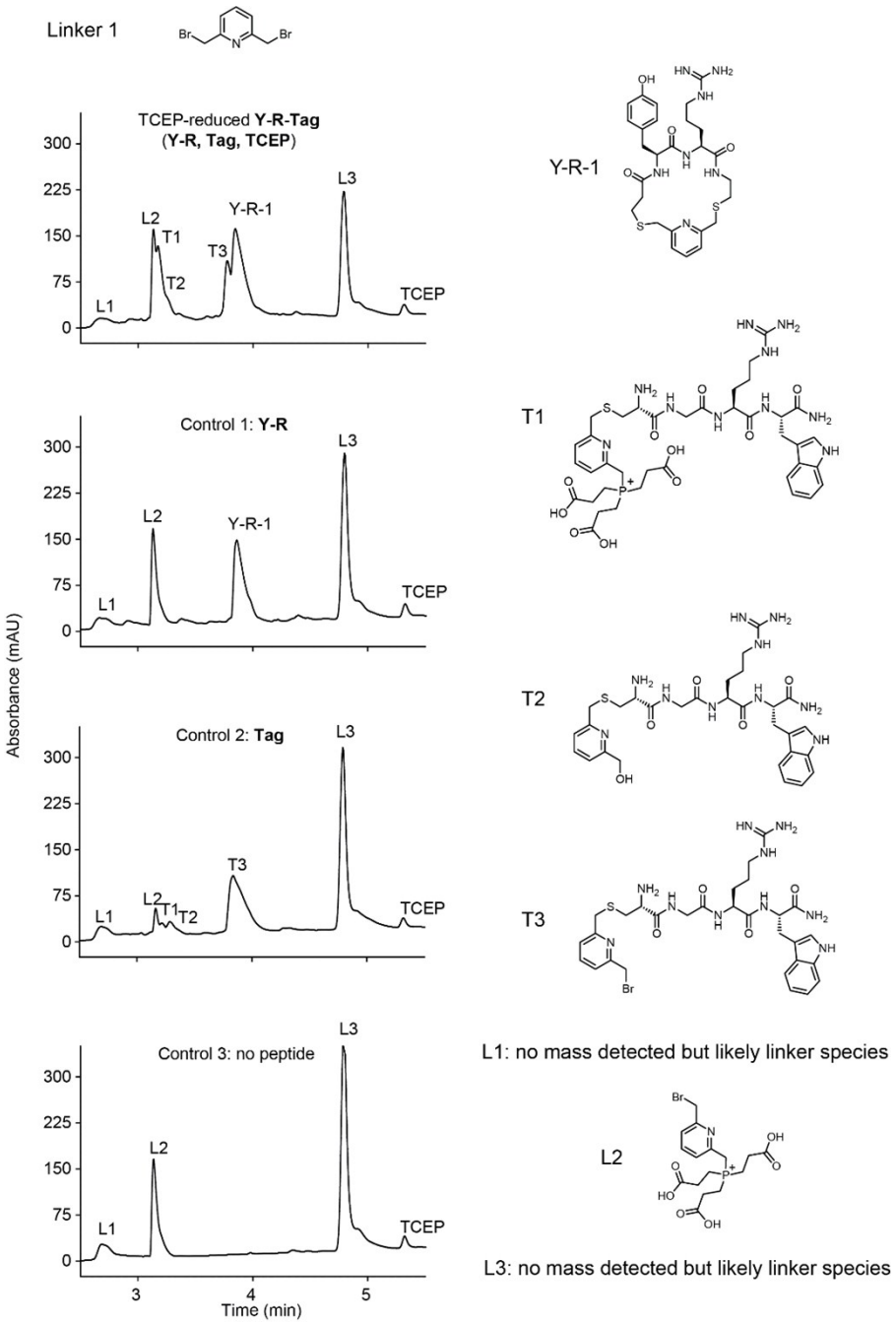


Figure S1. LC-MS analysis of the macrocyclization reactions of TCEP-reduced Y-R-Tag with reagents **1**-**5** (top chromatograms). The three chromatograms below show the analysis of control reactions: reaction of purified peptide Y-R with reagents **1**-**5**, reaction of purified Tag with reagents **1**-**5**, and reaction reagents without peptide. Peaks labeled with P1, P2, P3, L1,

L2, L3, T1, T2, T3 etc. are side products. Chemical structures that fit with their masses are indicated. Absorption was recorded at 220 nm.

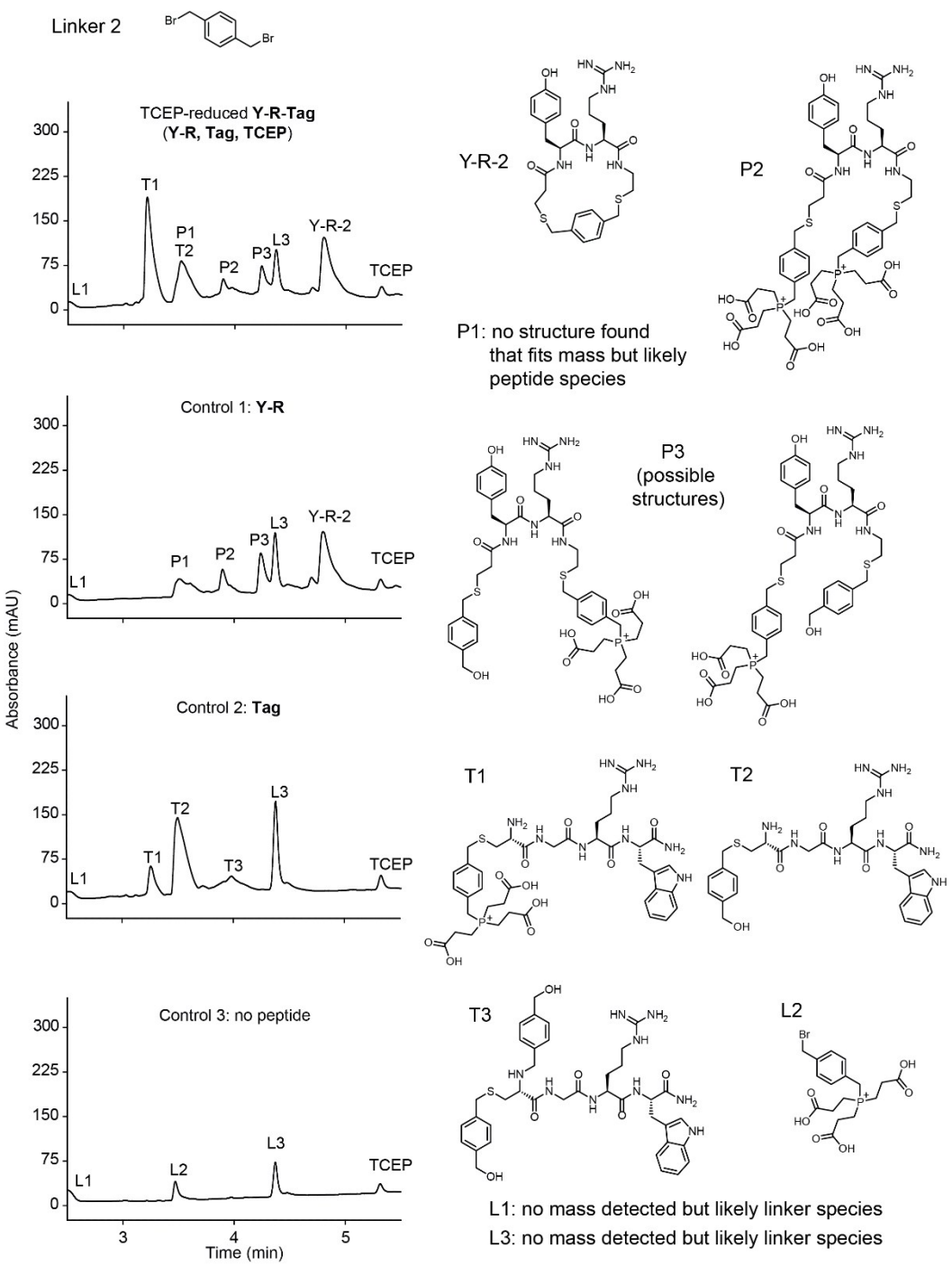
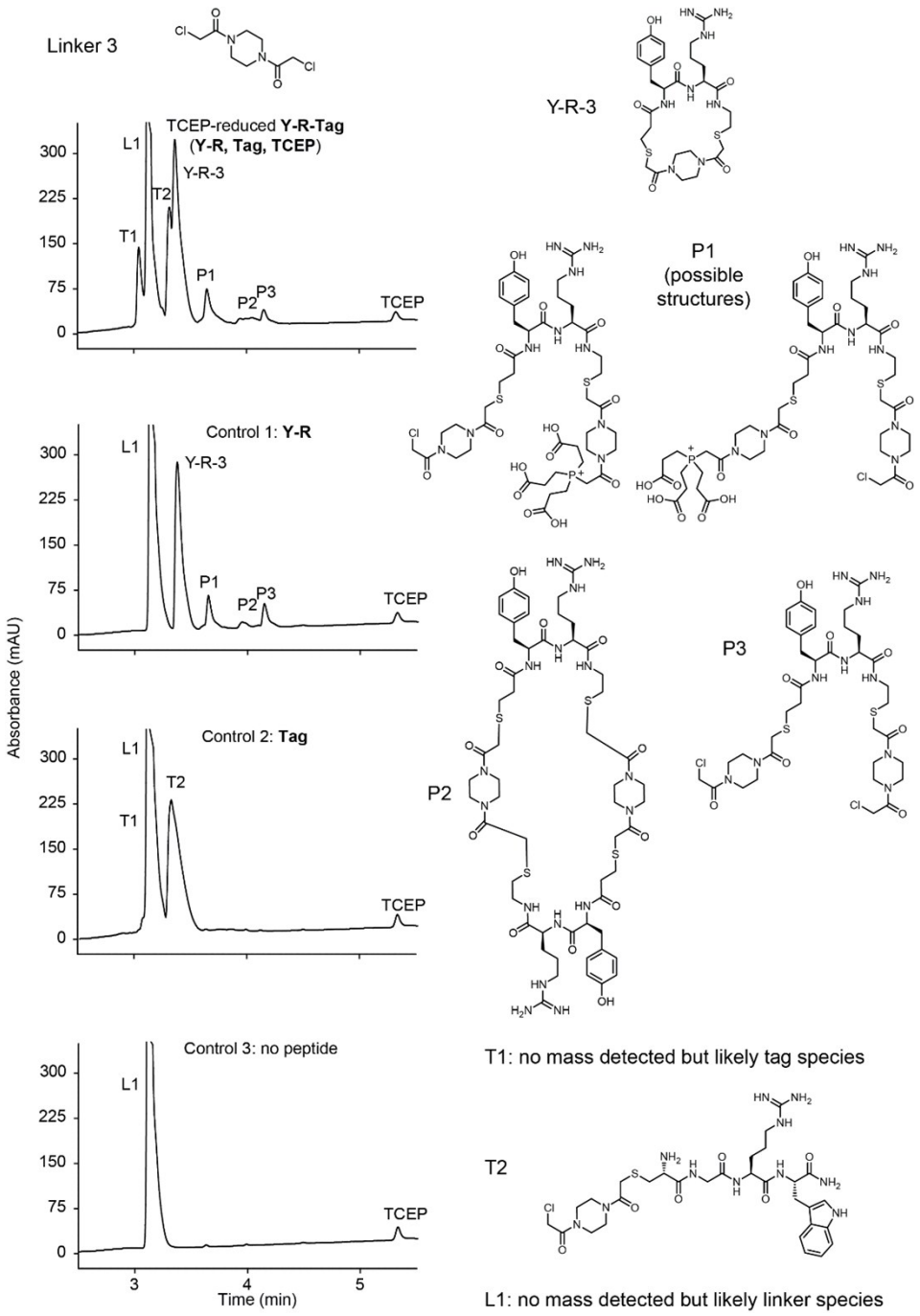


Figure S1. Continued



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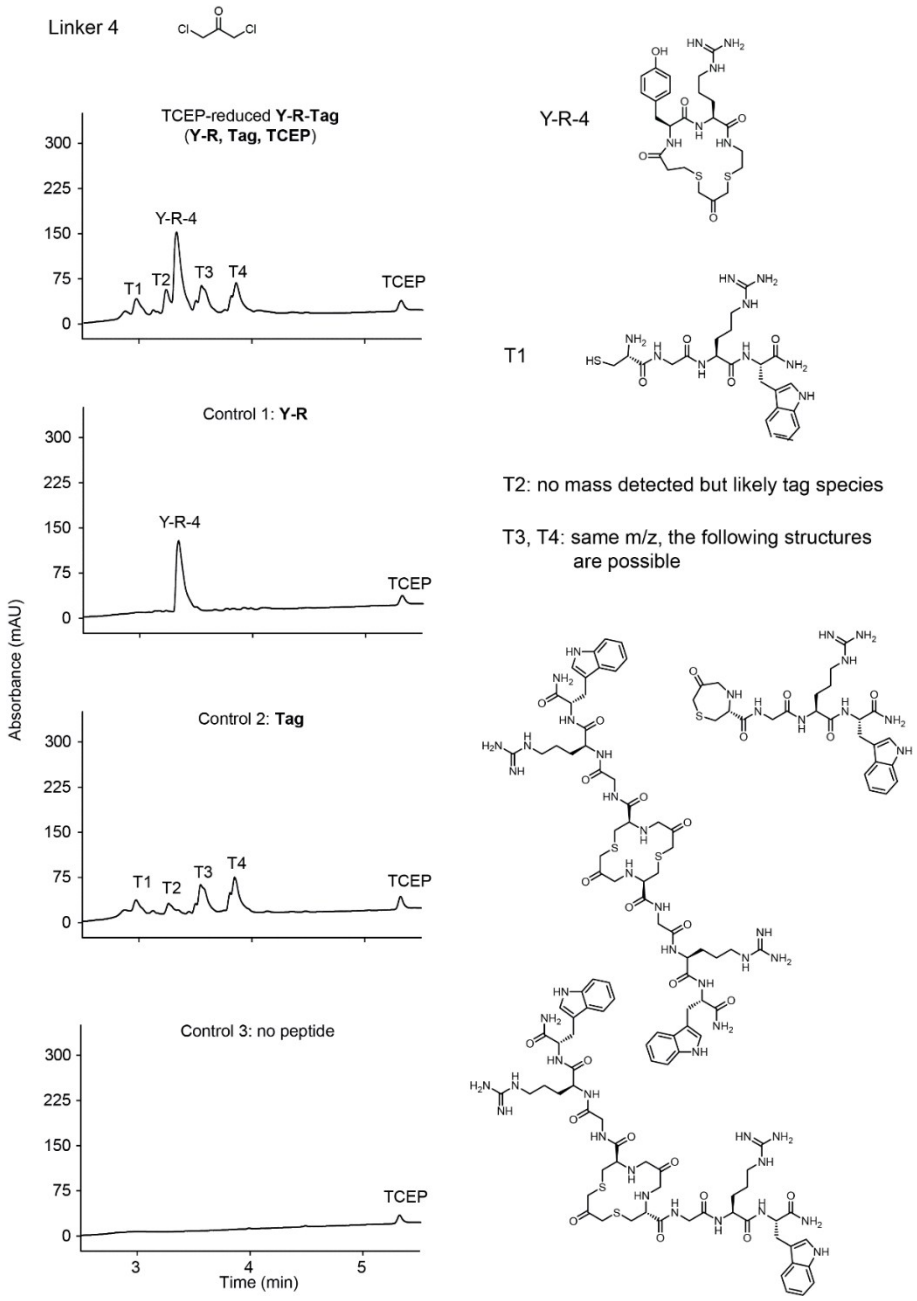


Figure S1. Continued

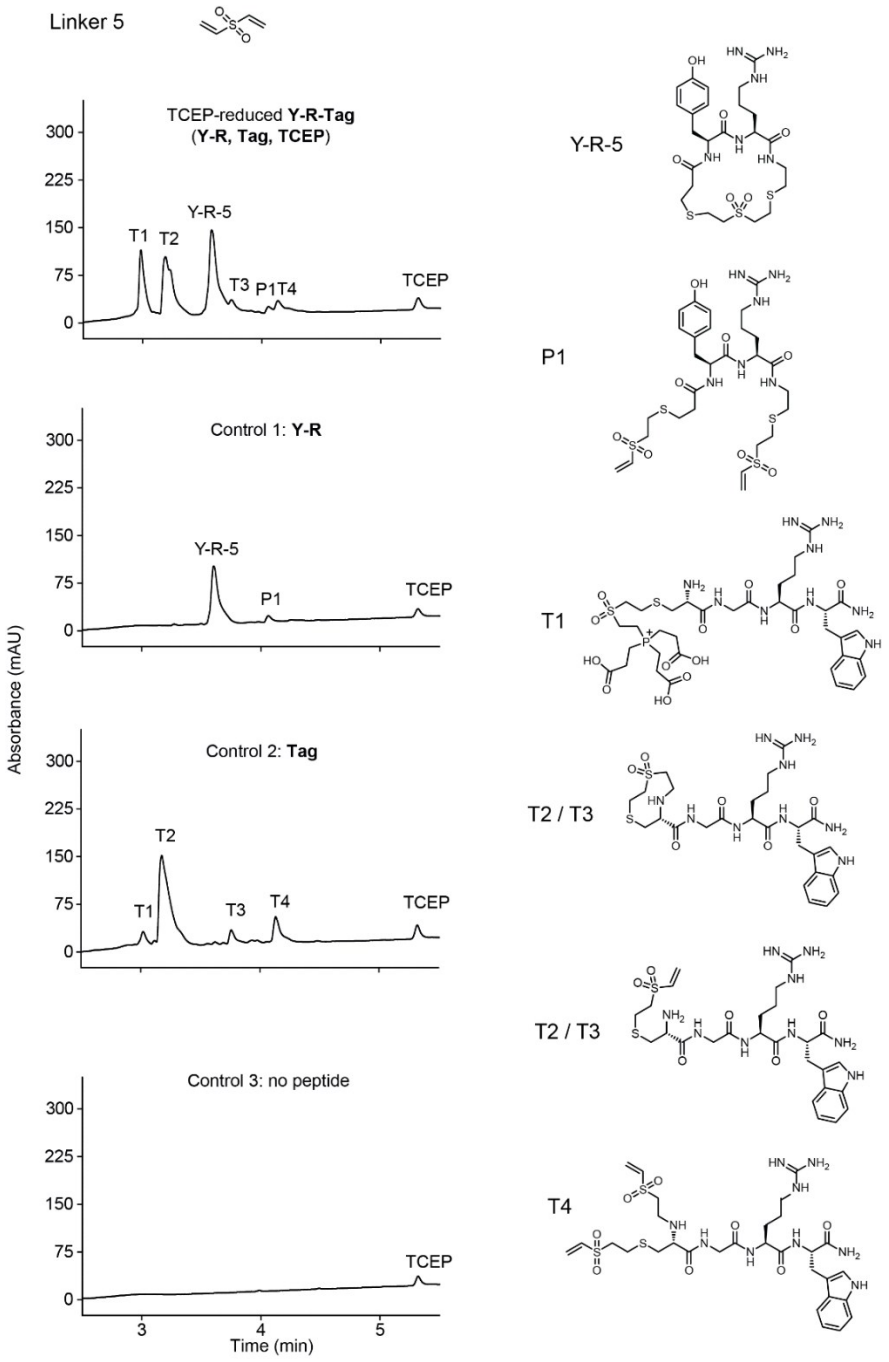


Figure S1. Continued

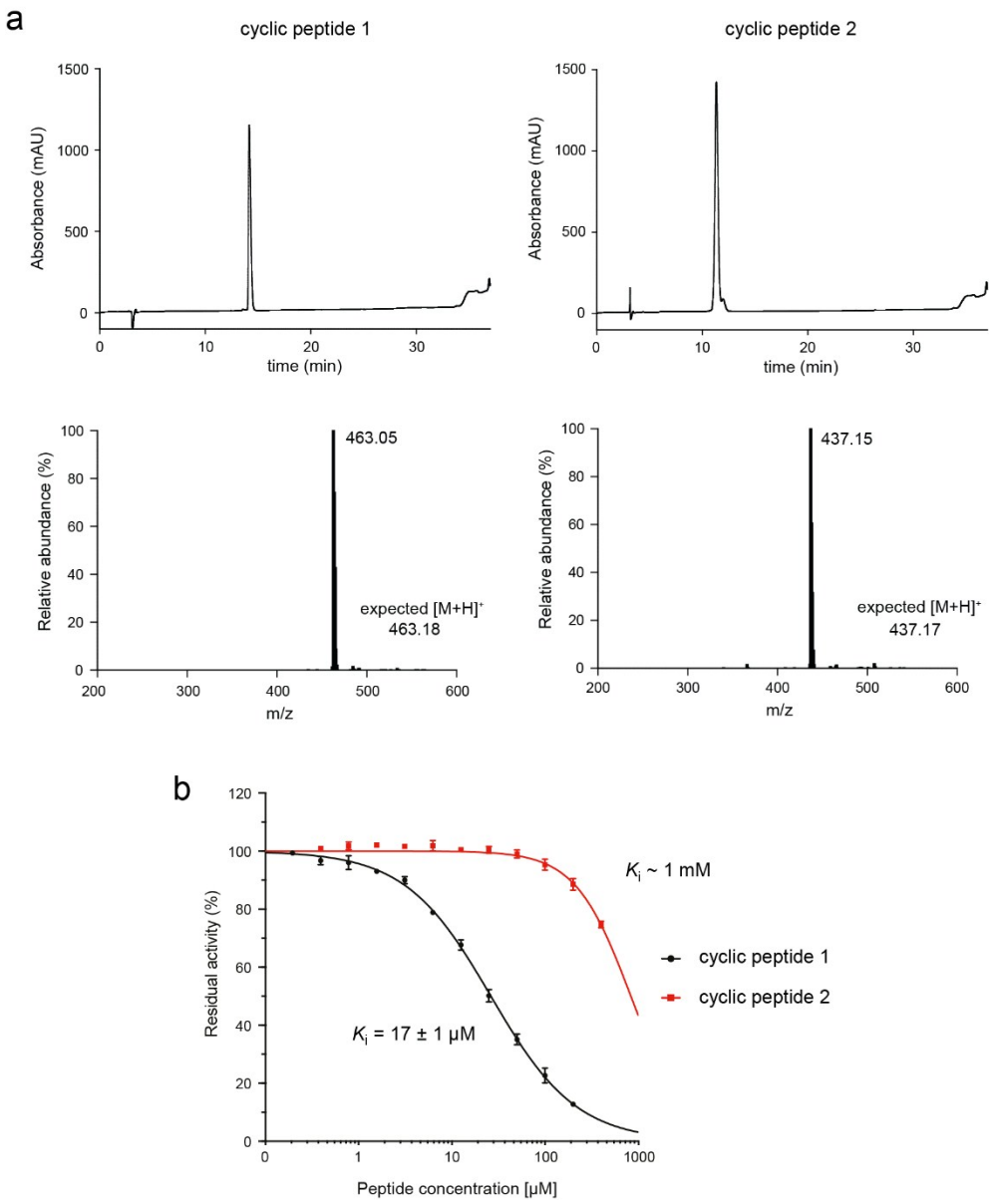
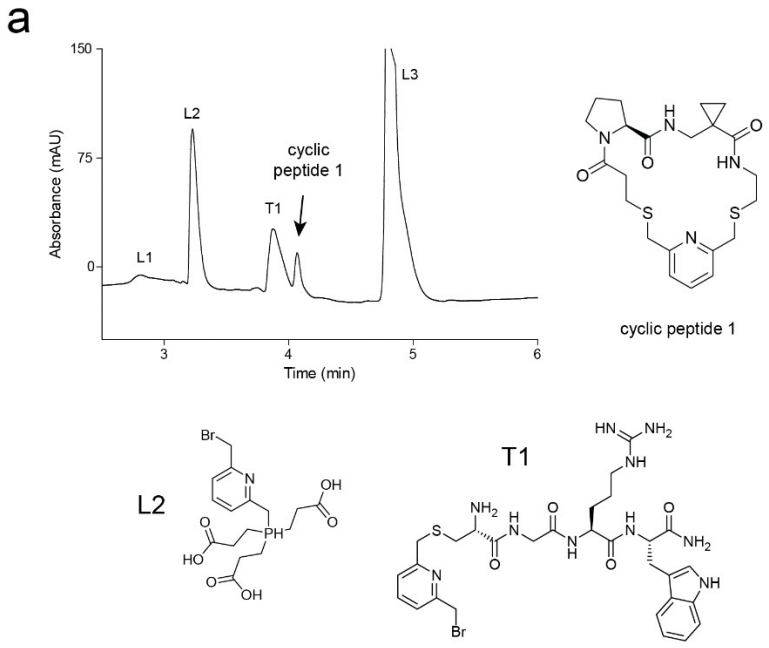
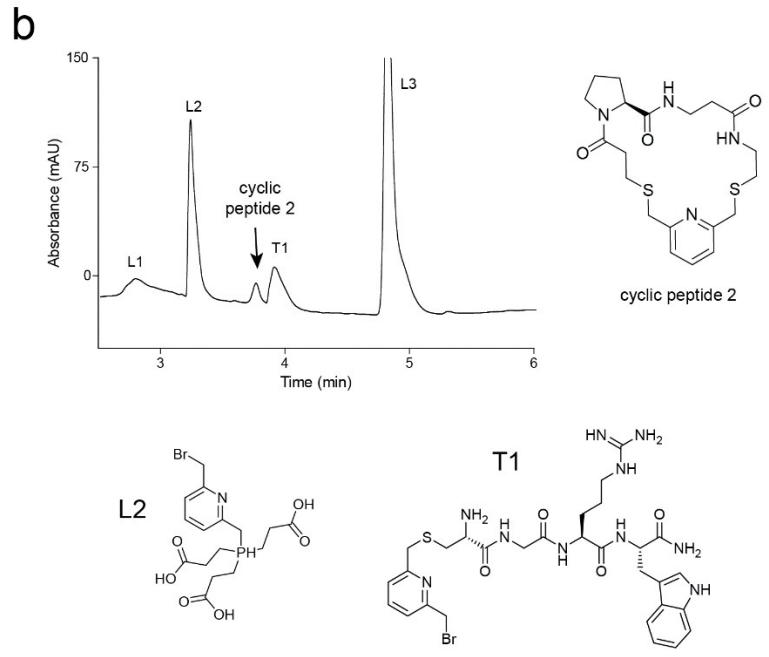


Figure S2. Standard peptides inhibiting thrombin with weak affinity. (a) Analysis of HPLC-purified peptide by analytical HPLC and MS. (b) Inhibition of human thrombin. Residual activity is shown for increasing cyclic peptide concentrations. Mean values and standard deviations for three measurements are shown.



L1, L3: no mass detected but likely linker species



L1, L3: no mass detected but likely linker species

Figure S3. Cyclic peptide thrombin inhibitors produced with the tag-based strategy. Analytical chromatograms of the cyclic peptide 1 (a) and cyclic peptide 2 (b) after diethyl ether-precipitation, tag release and macrocyclization with reagent **1** are shown. Side products identified based on MS analysis are indicated.