7 Experimental Procedures and Methods

7.1 Peptide Chemistry, Thermostability, and Self-Replication Experiments

7.1.1 General Procedures and Devices

Solvents, Chemicals, and Reagents

Diethylether for peptide precipitation was destilled and dried over $CaCl_2$. Water was triple destilled or purified on a MilliPore device. Guanidine hydrochloride, TCEP, disodium hydrogen phosphate, and sodium dihydrogen phosphate were purchased from Fluka. All other solvents, chemicals, and reagents are declared in the sections of the experimental procedures in which they were used. TCEP solutions were freshly prepared prior to use.

Solid Phase Peptide Synthesis

Peptides were synthesized on a semi-automatic peptide synthesizer ACT 90 (Advanced Chem Tech) in glas reactors with a volumetric capacity of 25, or 50mL, or on an automatic peptid synthesizer Syro XP (MultiSynTech) in 10mL polypropylene reactors. Solvents for peptide synthesis were commercially obtained “synthesis grade” and applied without any further purification (DMF, NMP), or have been dried over $CaCl_2$ and destilled. Fully protected amino acids were purchased from Iris Biotech, NovaBiochem, or MultiSynTech if not otherwise declared.

Mass Spectrometry

Molecular masses of synthesized pepides were determined via MALDI-ToF-MS (Matrix-Assisted Laser Desorption Ionisation – Time of Flight – Mass Spectrometry) on a mass spectrometer Voyager-DE^{TM} RP (PerSeptive Biosystems) using a nitrogen laser (λ=337nm). Peptides were crystalized with matrices 2,5-dihydroxybenzoic acid (Aldrich) or α-cyano-4-hydroxycinnamic acid (Sigma).

Analytical HPLC (High Performance Liquid Chromatography):

Qualitative analyses of peptides were performed on two different HPLC-devices:

1) Computer-controlled high-pressure-gradient LaChrom-HPLC-system (Merck-Hitachi), containing an Interface L-7000; two pumps L-7100 (one for each eluent); diode array detector L-7450; autosampler L-7200; solvent degasser L-7612; high-pressure-gradient mixer; Rheodyne injection valve 7725i, 20µL sample loop; administration of the device, data recording and analysis was performed with Chromatography Data Station Software version 4.0.

2) Computer-controlled low-pressure-gradient workstation (Thermo Separation Products, TSP), containing one pump P200 for both eluents with low-pressure-gradient mixer; autosampler P300; UV-detector UV100; solvent degasser L-7612 (Merck-Hitachi); different fluorescence detectors FL3000 (Thermo Separation Products), FP-920 (Jasco), RF-535 (Shimadzu); administration of the device, data recording and analysis was performed with software McDacq versin 1.5 (Bischoff).

The following reversed phase columns were used for analytical HPLC measurements:

C4: Trentec C4 (300) Reprosil-Pur 5µm (250mm x 4.6mm); Vydac C4 (300) 10µm (250mm x 4 mm); Vydac Protein C4 10µM (250mm x 4.0mm)

C8 : Capcell SG300C8 5µM (250mm x 4.0mm) ; Phenomenex Luna C8(2) 5µM (250mm x 4,6mm); Phenomenex Luna C8(2) 10µM (250mm x 4.6mm);

C18 : Capcell SG120C18 5µM (250mm x 4.6mm); Knauer Europrep 60-C18 10µM (250mm x 4,0mm); Knauer Nucleosil 100-C18 5µM (250mm x 4.0mm); Phenomenex Luna C18 5µM (250mm x 4.6mm)

HPLC runs were performed with a flow rate of 1 mL/min applying eluent gradients. Eluent A was 0.1% TFA (uvasol for spectroscopy, Merck), 5% ACN, 94.9% H_2O; eluent B was 0.1% TFA (uvasol for spectroscopy, Merck), 5% H_2O, 94.9% ACN. Eluents were degassed prior to use. UV-detection occured at λ_{ab}=220nm, fluorescence detection at λ_{ex}=230nm (exitation) and λ_{em}=310nm (emission).

Semipreparative HPLC:

Purification of peptides in a semipreparative scale was performed on a high-pressure-gradient HPLC-device (Knauer), containing HPLC programmer 50; two pumps 64 (one for each eluent); variable wavelength detector; plotter; injection valve 7125; 1mL sample loop. Reversed phase columns Vydac Protein C4 10µm (250mm x 10mm) and Merck LiChroCart C18 5µm (250mm x 10mm) were used. HPLC runs were performed with a flow rate of 4-5 mL/min applying eluent gradients. Eluent A was 0.1% TFA (uvasol for spectroscopy, Merck), 5% ACN, 94.9% H_2O; eluent B was 0.1% TFA (uvasol for spectroscopy, Merck), 5% H_2O, 94.9% ACN. UV-detection occured at λ_{ab}=220nm.

Preparative HPLC:

Purification of peptides in a preparative scale was performed on two different HPLC-devices:

1) Computer-controlled high-pressure-gradient system LKB Bromma (Pharmacia), containing central processor LC2252; two pumps 2248 (one for each eluent); two-wavelength UV-detector 2141; solvent degasser; motor valve PMV-7; high-pressure-gradient mixer; 1mL and 5mL sample loops.

2) Knauer high-pressure-gradient system, containing interface Smartline Manager 5000 with solvent degasser; two pumps Smartline pump 1000 (one for each eluent); variable UV-detector 2500; injection valve; high-pressure-gradient mixer; 1mL and 5mL sample loops.

Reversed phase columns Vydac Protein C4 10µm (250mm x 22mm) and Phenomenex Luna C8 10µm (250mm x 21.2mm) were used. HPLC runs were performed with a flow rate of 20mL/min applying eluent gradients. Eluent A was 0.1% TFA (uvasol for spectroscopy, Merck), 5% ACN, 94.9% H_2O; eluent B was 0.1% TFA (uvasol for spectroscopy, Merck), 5% H_2O, 94.9% ACN. UV-detection occured at λ_{ab}=220nm.

CD-Spectroscopy:

CD-spectroscopy was performed on a Jasco J-715 spectropolarimeter. Tempering of the samples was carried out using a Jasco PTC-348WI peltier thermostat. Administration of the device and data analysis was performed with the software J-700.

7.1.2 Peptide Synthesis

All peptides were synthesized from the C-terminal to the N-terminal end on solid support, applying Fmoc/*tert*-butyl protecting group strategy. The following protected derivatives of standard canonical amino acids were used for the synthesis of all peptides:

Fmoc-Arg(Pbf)-OH (arginine), Fmoc-Cys(Trt)-OH (cysteine), Fmoc-Glu(OtBu)-OH (glutamic acid), Fmoc-Leu-OH (leucine), Fmoc-Lys-OH (lysine), Fmoc-Ser(tBu)-OH (serine), Fmoc-Tyr(tBu)-OH (tyrosine).

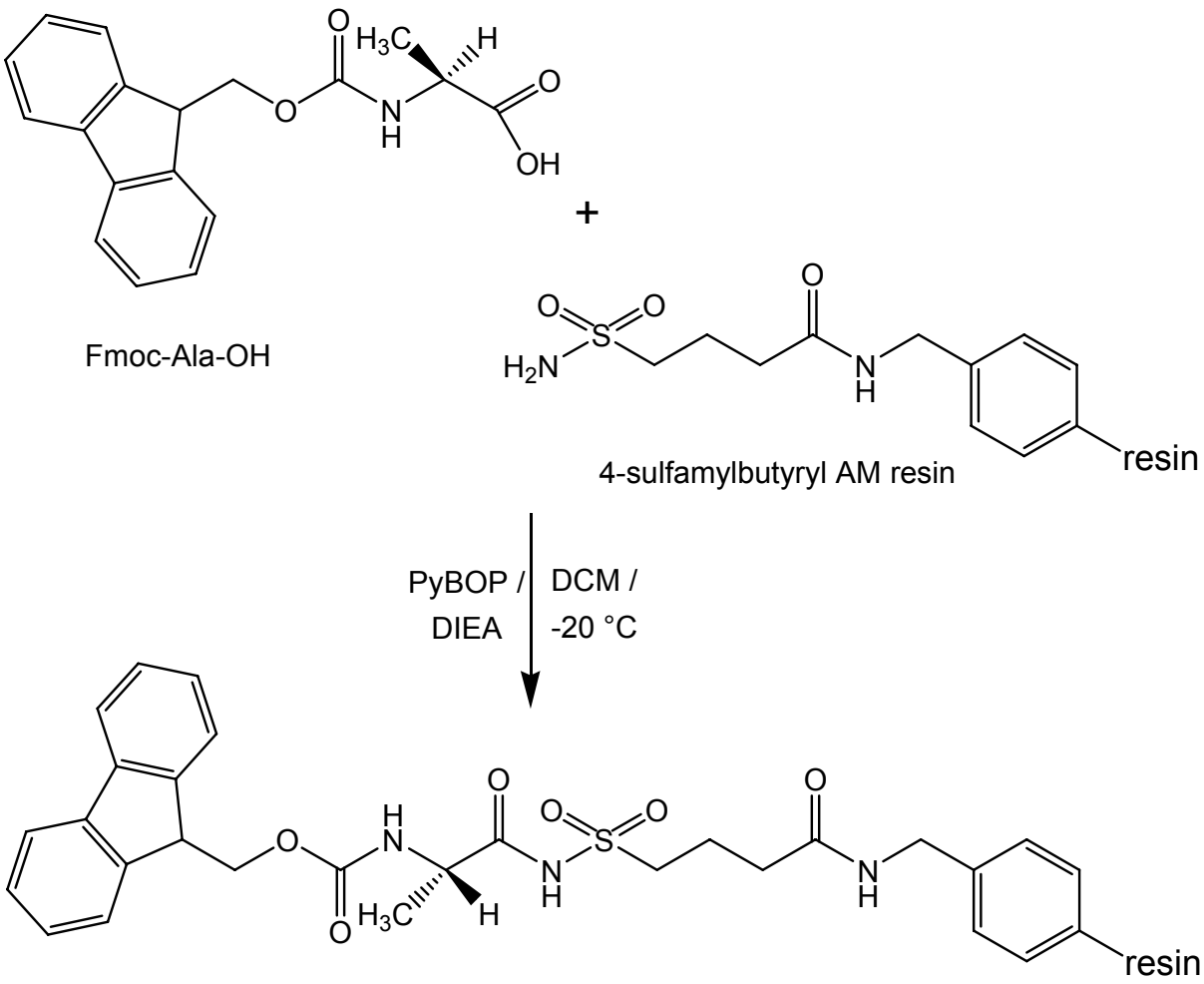
Fmoc-amino acids were purchased from Iris Biotech, NovaBiochem, or MultiSynTech if not otherwise declared. Biotin was obtained from Sigma. Peptide coupling reagents were obtained from Acros (DIEA), NovaBioChem / MultiSynTech (HOBt, HBTU, TBTU, TCTU), Sigma (DIC), and PerSeptive Biosystems (HATU, HOAt). NMM was obtaind from Fluka.

7.1.2.1 Coupling of the First Amino Acid to the Solid Support

The nucleophilic peptide fragment (N) and the non-substituted electrophilic peptide fragment possessing a C-terminal carboxyl group (E_C) were synthesized on Wang-resins, which were purchased (NovaBiochem), having the C-terminal amino acid of the peptide already linked to the solid support. These resins (Fmoc-Glu(OtBu)-Wang in case of N and Fmoc-Ala-Wang in case of E_C) could be used for peptide chain elongation as obtained.
The electrophilic peptide fragments with a C-terminal benzylthioester were synthesized according to the “safety-catch” strategy.[233] The C-terminal amino acid of these peptides, which is alanine in all cases, was linked to 4-sulfamylbutyryl AM resin (NovaBiochem) via *in situ* activation of the carboxyl group and subsequent amide bond formation (Scheme 7.1). The resin-bound acylsulfonamide was then completely stable to basic or strongly nucleophilic reagents and, therefore compatible with Fmoc SPPS.

3g of 4-sulfamylbutyryl AM resin (maximum loading capacity: 1.12 mmol/g) were swelling in 20mL DMF for 10min. DMF was removed, and the procedure was repeated two times. Resin was resuspended in 100mL DMF and cooled down to -20°C. 4mmol Fmoc-Ala-OH, 4mmol PyBOP (NovaBiochem), and 8mmol DIEA were added in 4 portions in 60min intervals. After 8h of reaction under agitation (no stirring) at -20°C, additional 1mmol PyBOP and 1mmol DIEA were given to the reaction. Coupling proceeded for further 12h at room temperature. The resin was filtered and washed three times with each of the solvents DMF, DCM, and

diethylether. The resin loading procedure was repeated as described with 3mmol Fmoc-Ala-OH, 3mmol PyBOP, and 6mmol DIEA. After washing, the resin was dried in vacuo.



"safety-catch" resin loaded with Fmoc-Ala

Scheme 7.1: *Coupling of Fmoc-Ala-OH to 4-sulfamylbutyryl AM resin.*

The loading of the resin was determined via UV-detection of the Fmoc protecting group. 1mg loaded resin were rocked for 3min in 3mL 20% piperidine in DMF. The extinction of the supernatant of the resin suspension was measured at 290nm. The procedure was performed three times and a mean value of extinction was determined. Resin loading was calculated via the following equation:

$$\text{Fmoc-loading [mmol/g]} = E / (1.65 \times m_{resin}\ [mg])$$

A resin loading of 0.65mmol/g was determined for Fmoc-Ala loaded 4-sulfamylbutyryl resin. To prepare for peptide synthesis, one portion of the loaded resin was treated with acetanhydride for capping of non-loaded sulfamyl groups on the resin. After swelling for

30min in 10mL DMF, 1.5 g resin were filtered and rocked in 10mL 10% acetic anhydride / 10% DIEA in DMF for 10min. Resin was filtered and reaction was repeated two times. After capping, resin was washed several times with DMF and finally with DCM. The resin was dried in vacuo and stored at -20°C.
Later couplings of Fmoc-Ala-OH to the 4-sulfamylbutyryl resin were performed, following this protocol and resulted in differing loading values.

7.1.2.2 Peptide Chain Elongation

The nucleophilic peptide fragment (N, 0.2mmol) and the electrophilic peptide fragments (E_X, 0.1 – 0.2mmol) bearing a C-terminal benzylthioester were synthesized on a semi-automatic peptide synthesizer, the carboxyl-derivative of the non-substituted electrophilic peptide fragment (E_C, 0.1mmol) was synthesized on an automatic peptide synthesizer (Section 7.1.1). Reagent equivalents refer to the amount of peptide / amino acid coupled to the resin that was used in the reaction. Amino acid coupling procedures that differ from the protocol described below are depicted separately for each peptide in section 7.1.2.5. Reaction cylcles started with Fmoc-deprotection, followed by washing and amino acid coupling. Prior to first coupling cycle, swelling of the resins were perfomed three times in 10mL DMF for 10min.

Cleavage of the Fmoc Protecting Group

Semi-automatic peptide syntheses: Resin was rocked with 10mL 2% DBU / 2% piperidine in DMF for 20min. After reaction, resin was filtered, one time washed with 10mL DMF for 1min and rinsed with additional 10mL DMF. The procedure was repeated one time. For washing, resin was washed with 10mL DMF for 1min and rinsed with additinal 10mL DMF. Washing procedure was repeated five times.

Automatic peptide syntheses: Resin was mixed with 2mL 40% piperidine in DMF and reaction proceeded for 10min with agitation in defined intervals. Deprotection was repeated once with 2mL 20% piperidine in DMF, followed by six times washing with 2.5 mL DMF for 1min each.

Coupling of the Amino Acid

Semi-automatic peptide syntheses: Three equivalents of α-amino-Fmoc-protected and fully side chain-protected amino acid were solved in 10mL DMF together with 3 equivalents of each HOBt and a guanidinium salt *in situ* coupling reagent (TBTU, HBTU, or TCTU). Reaction started with addition of 6 equivalents of DIEA and proceeded under agitation for 30min. After reaction, resin was filtered, washed with 10 mL DMF for 1min and rinsed with additional 10mL DMF. The amino acid coupling procedure was repeated once, followed by 3 washing steps (washing for 1min with 10mL DMF and rinsing with 10mL DMF).

Automatic peptide syntheses: Coupling reactions were performed in 2.4mL DMF, containing 4 equivalents of each α-amino-Fmoc-protected and fully side chain-protected amino acid, HOBt, and HCTU. Reactions were started with addition of 8 equivalents DIEA and proceeded for 30min with agitation in defined intervals. After washing the resin with 2.6mL DMF for 1min, amino acid coupling reaction was repeated once. Prior to Fmoc-deprotection of the next coupling cycle, the resin was washed 3 times with 2.6mL DMF for 1min each.

Coupling of biotin to the N-terminal amino acid of the electrophilic peptide fragments was performed similarly to the standard coupling procedure with the following exceptions: Only 2 equivalents of each biotin, HOBt, and the *in situ* coupling reagent and 4 equivalents of DIEA were used. Solvation of biotin in DMF was performed with ultrasound. Reaction occured 2 times for 60min. Coupling of biotin to E_C was performed in a 10mL polypropylene syringe, equipped with a bottom polyethylene filter.

Peptide chain elongation processes ended with an Fmoc-deprotection step. After last amino acid coupling cycle, the resin was washed 3 times with DCM. Electrophilic peptide fragments were additionally washed 3 times with NMP.

Capping of Free α-Amino Groups

The capping of free α-amino groups was performed after amino acid coupling reactions only if mentioned in the detailed descriptions of the individual peptides (Section 7.1.2.5). Therefore, the resin was rocked with 10mL 10% acetic anhydride / 10% DIEA in DMF for 10min. The resin was filtered and procedure was repeated 2 times. After capping, the resin was washed 3 times with DMF (washing with 10mL DMF for 1min, followed by rinsing with additional 10mL DMF).

7.1.2.3 Deprotection and Cleavage from Solid Support

Side chain deprotection and resin cleavage of peptides synthesized on Wang-resin (N, E_C) was accomplished simultaneously under strong acidic conditions. Peptides synthesized on 4-sulfamylbutyryl resin were first cleaved from the resin, combined with formation of C-terminal benzylthioesters. Subsequently, acid-labile side chain protecting groups were removed. Precipitation of the peptide was followed by analyses and purification. TFA, EDT, and TIS were purchased from Acros, iodoacetonitrile, benzylmercaptan, and sodium thiophenate from Fluka.

Side Chain Deprotection and Cleavage from Wang Resin

Nucleophilic peptide fragment N: Reaction was performed in a 25mL glass reactor with bottom filter. Resin was rocked with 8.5mL TFA, 0.5mL H_2O, 0.5mL EDT, and 0.5mL TIS for 4h. After reaction, peptide solution was discharged. Resin was washed 3 times with 6mL DMF and discharged solutions were pooled with the first peptide fraction in a 50mL round-bottom flask. DCM and TFA were evaporated and peptide was precipitated with approx. 50mL ice cold diethylether. Precipitation occured over night at -20°C. Peptide was filtered, washed several times with ice cold diethylether, and dried in vacuo.

Carboxyl-derivative of the non-substituted electrophilic peptide fragment E_C: Reaction was performed in a 10mL polypropylene syringe with a bottom polyethylene filter. Resin was rocked with 2.7mL TFA, 50µL H_2O, and 0.3mL TIS for 3.5h. After reaction, peptide was precipitated with 12mL ice cold diethylether in a 15mL polypropylene tube. After precipitation for 30min at -20°C, peptide was washed 7 times with approx. 10mL ice cold diethylether and dried in vacuo.

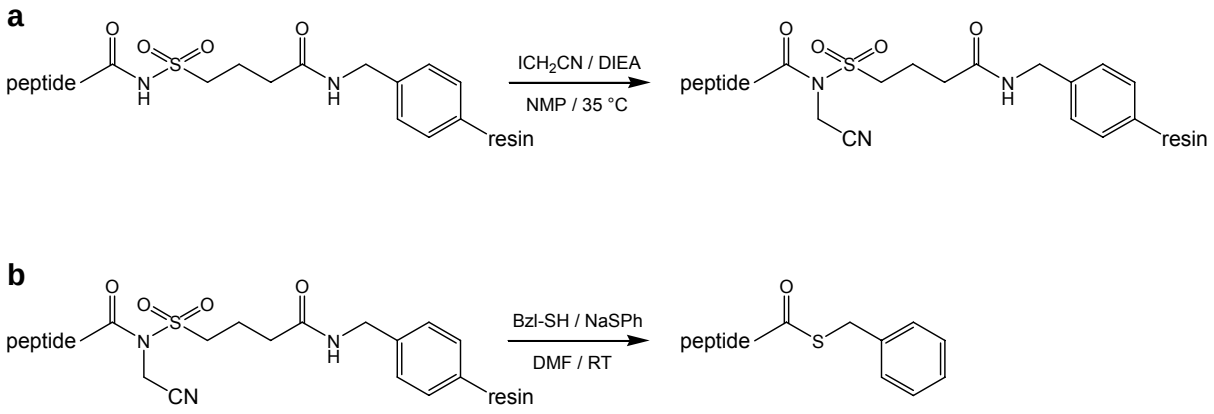
Side Chain Deprotection and Cleavage from 4-sulfamylbutyryl Resin

Cleavage from 4-sulfamylbutyryl resin occured in two steps. Activation of the acylsulfonamide moiety was followed by thioester formation and release of the peptide from the solid support (Scheme 7.2). Reagent equivalents refer to the amount of peptide coupled to the resin.

Activation via N-alkylation: Activation reactions were performed in polypropylene reactors with polyethylene bottom filters. 5mL NMP containing 25 equivalents iodoacetonitrile (Fluka) and 11 equivalents DIEA were added to the resin and reaction proceeded approx. 3h at 35°C

under exclusion of light. Reactions were repeated 2 times for approx. 15h and 6h, respectively. In between, resin was washed 2 times with 5mL NMP each. After alkylation reaction, resin was washed 3 times with 8mL DMF each.

Cleavage from solid support: 5mL DMF containing 10 equivalents benzylmercaptan and 0.25 equivalents sodium thiophenate were added to the washed resin, and cleavage reaction proceeded for approx. 15h. Peptide solution was saved in a 50mL round-bottom flask. Reactions were repeated once with 30 equivalents benzylmercaptan and 0.5 equivalents sodium thiophenate in 5mL DMF, resins were washed twice with 5mL DMF and peptide fractions were pooled. DMF was evaporated until the side-chain protected pepides were completely dry.



Scheme 7.2: *Activation of the acylsulfonamide via N-alkylation (a) and cleavage of the peptide from the solid support as benzylthioester (b). NaSPh was added in catalytic amounts to initially cleave the peptide as α-phenyl-thioester, followed by an exchange in situ with an large excess of benzylmercaptan.*

Side chain-deprotection: Peptides were solved in 9mL TFA, 0.5mL H_2O and 0.5mL TIS, and deprotection reactions proceeded for approx. 4h. TFA was evaporated and peptides were precipitated with approx. 50mL ice cold diethylether. After precipitation at -20°C over night, peptides were filtered, washed several times with ice cold diethylether and dried in vacuo.

7.1.2.4 Analysis and Purification

The dry peptides were solved in 2-5mL 0.1% TFA in acetonitrile / water (1:1) and lyophyllized after evaporation of acetonitrile and traces of diethylether. After analyses of peptide purity via

analytical HPLC, preferably using a LaChrom HPLC-system with diode array detection (Section 7.1.1), and verification of peptide identity via mass spectrometry of separated HPLC peaks, different portions of the peptides were purified via preparative HPLC. Selected fractions of each purification were analyzed via analytical HPLC, and those showing pure peptide were pooled. Acetonitrile was evaporated and pure peptides were lyophyllized. The amounts of crude peptide that were purified depended on how much pure peptide was needed. A maximum of 50mg peptide were purified per HPLC-run. Both preparative HPLC systems as well as the semipreparative HPLC device were used for purification of peptides. Acetonitrile was purchased from Merck or Acros and used without any further purification.

7.1.2.5 Detailed Description of Peptide Syntheses

CLKYELRKLEYELKKLEYELSSLE (N):

The peptide (0.2mmol) was synthesized on Wang resin, that was preloaded with Fmoc-Glu(OtBu)-OH (0.54mmol/g). For purification, peptide was solved in 0.1% TFA / 30% ACN / 69.9% H_2O.

Analytical HPLC: 0% - 100% eluent B in 30min (gradient)
Vydac Protein C4 (column)

Preparative HPLC: 0min – 15min: 30% eluent B (isocratic)
15min – 45min: 30% - 70% B (gradient)
Vydac Protein C4 (column)

Molecular Mass: 3020.5 Da (calculated average mass)
3021.3 Da (detected mass)

Bio-RLEELREKLESLRKKLA (E_C):

The peptide (0.1mmol) was synthesized on Wang resin, that was preloaded with Fmoc-Ala-OH (0.67 mmol/g). For purification, peptide was solved in 0.1% TFA / 5% ACN / 94.9% H_2O.

Analytical HPLC: 0% - 100% eluent B in 30min (gradient)
Phenomenex C8 / 10µm (column)

Preparative HPLC: 0min – 30min: 0% - 70% B (gradient)

Phenomenex C8 / 10µm (column)

Molecular Mass: 2337.5 Da (calculated average mass)
2338.2 Da (detected mass)

Bio-RLEELREKLESLRKKLA-(S-Bzl) (E_o):

The peptide (0.2mmol) was synthesized on 4-sulfamylbutyryl resin, that was loaded with Fmoc-Ala-OH (0.65 mmol/g). For purification, peptide was solved in 0.1% TFA / 25% ACN / 74.9% H_2O.

Analytical HPLC: 0% - 100% eluent B in 30min (gradient)
Vydac Protein C4 (column)

Preparative HPLC: 0min – 15min: 25% eluent B (isocratic)
15min – 45min: 25% - 70% B (gradient)
Vydac Protein C4 (column)

Molecular Mass: 2443.7 Da (calculated average mass)
2447.3 Da (detected mass)

Bio-RLEELREKEESLRKKLA-(S-Bzl) (E_{L9E}):

The peptide (0.1mmol) was synthesized on 4-sulfamylbutyryl resin, that was loaded with Fmoc-Ala-OH (0.5 mmol/g). For purification, peptide was solved in 0.4% TFA / 20% ACN / 79.6% H_2O.

Analytical HPLC: 0% - 100% eluent B in 30min (gradient)
Vydac Protein C4 (column)

Preparative HPLC: 0min – 15min: 20% eluent B (isocratic)
15min – 45min: 20% - 60% B (gradient)
Vydac Protein C4 (column)

Molecular Mass: 2459.7 Da (calculated average mass)
2459.0 Da (detected mass)

Bio-RLEELREALESLRKKLA-(S-Bzl) (E_{K8A}):

The peptide (0.1mmol) was synthesized on 4-sulfamylbutyryl resin that was loaded with Fmoc-Ala-OH (0.65mmol/g). For purification, peptide was solved in 0.1% TFA / 25% ACN / 74.9% H_2O.

Analytical HPLC: 0% - 100% eluent B in 30min (gradient)
Vydac Protein C4 (column)

Preparative HPLC: 0min – 15min: 25% eluent B (isocratic)
15min – 45min: 25% - 70% B (gradient)
Vydac Protein C4 (column)

Molecular Mass: 2386.6 Da (calculated average mass)
2391.2 Da (detected mass)

Bio-RLEELREKAESLRKKLA-(S-Bzl) (E_{L9A}):

The peptide (0.1mmol) was synthesized on 4-sulfamylbutyryl resin, that was loaded with Fmoc-Ala-OH (0.65mmol/g). For purification, peptide was solved in 0.1% TFA / 20% ACN / 79.9% H_2O.

Analytical HPLC: 0% - 100% eluent B in 30min (gradient)
Vydac Protein C4 (column)

Preparative HPLC: 0min – 15min: 20% eluent B (isocratic)
15min – 45min: 20% - 60% B (gradient)
Vydac Protein C4 (column)

Molecular Mass: 2401.6 Da (calculated average mass)
2402.2 Da (detected mass)

Bio-RLEELREEGlyLESLRKKLA-(S-Bzl) (E_{K8EGly}):

The peptide (0.15mmol) was synthesized on 4-sulfamylbutyryl resin, that was loaded with Fmoc-Ala-OH (0.65mmol/g). Fmoc-protected *(S)*-ethylglycine (EGly) was purchased from Bachem. The non-natural amino acid was coupled two times for 60min with 2.5 equivalents

Fmoc-EGly-OH, 2.5 equivalents HOBt, 2.5 equivalents TCTU, and 5 equivalents DIEA in each coupling. For purification, peptide was solved in 0.1% TFA / 20% ACN / 79.9% H_2O.

Analytical HPLC: 0% - 100% eluent B in 30min (gradient)
Vydac Protein C4 (column)

Preparative HPLC: 0min – 15min: 20% eluent B (isocratic)
15min – 45min: 20% - 60% B (gradient)
Vydac Protein C4 (column)

Molecular Mass: 2400.6 Da (calculated average mass)
2407.6 Da (detected mass)

Bio-RLEELREKEGlyESLRKKLA-(S-Bzl) (E_{L9EGly}):

The peptide (0.15mmol) was synthesized on 4-sulfamylbutyryl resin, that was loaded with Fmoc-Ala-OH (0.65mmol/g). Fmoc-protected *(S)*-ethylglycine (EGly) was purchased from Bachem. The non-natural amino acid was coupled 2 times for 60min with 2.5 equivalents Fmoc-EGly-OH, 2.5 equivalents HOBt, 2.5 equivalents TCTU, and 5 equivalents DIEA in each coupling. For purification, peptide was solved in 0.1% TFA / 20% ACN / 79.9% H_2O.

Analytical HPLC: 0% - 100% eluent B in 30min (gradient)
Vydac Protein C4 (column)

Preparative HPLC: 0min – 15min: 20% eluent B (isocratic)
15min – 45min: 20% - 60% B (gradient)
Vydac Protein C4 (column)

Molecular Mass: 2415.6 Da (calculated average mass)
2422.7 Da (detected mass)

Bio-RLEELREDfeGlyLESLRKKLA-(S-Bzl) ($E_{K8DfeGly}$):

The peptide (0.1mmol) was synthesized on 4-sulfamylbutyryl resin, that was loaded with Fmoc-Ala-OH (0.65mmol/g). *(S)*-4,4-difluoroethylglycine was synthesized in the group of Prof. Beate Koksch, according to a published strategy[234] and Fmoc-protected with Fmoc-Cl, applying a standard protocol. The non-natural amino acid was coupled twice for 10h and

13h, respectively, in 7mL DMF with 2 equivalents of each Fmoc-DfeGly-OH, HOAt, and DIC in both coupling steps. Fmoc-Glu(OtBu)-OH in amino acid position 7 was coupled twice for 17h and 8h, respectively, in 7mL DMF with 3 equivalents of each amino acid, HOAt, and DIC. Prior to Fmoc-deprotection of Glu7, free α-amino groups were capped. For purification, peptide was solved in 0.1% TFA / 50% ACN / 49.9% H_2O.

Analytical HPLC: 0% - 100% eluent B in 30min (gradient)
Capcell C8 (column)

Preparative HPLC: 0min – 15min: 20% eluent B (isocratic)
15min – 45min: 20% - 60% B (gradient)
Vydac Protein C4 (column)

Molecular Mass: 2436.6 Da (calculated average mass)
2439.6 Da (detected mass)

Bio-RLEELREKDfeGlyESLRKKLA-(S-Bzl) ($E_{L9DfeGly}$):

The peptide (0.1mmol) was synthesized on 4-sulfamylbutyryl resin, that was loaded with Fmoc-Ala-OH (0.65mmol/g). *(S)*-4,4-difluoroethylglycine was synthesized in the group of Prof. Beate Koksch, according to a published strategy and Fmoc-protected with Fmoc-Cl, applying a standard protocol. The non-natural amino acid was coupled twice for 10h and 13h, respectively, in 7mL DMF with 2 equivalents of each Fmoc-DfeGly-OH, HOAt, and DIC in both coupling steps. Fmoc-Lys(Boc)-OH in amino acid position 8 was coupled twice for 17h and 8h, respectively, in 7mL DMF with 3 equivalents of each amino acid, HOAt, and DIC. Prior to Fmoc-deprotection of Lys8, free α-amino groups were capped. For purification, peptide was solved in 1% TFA / 40% ACN / 59% H_2O.

Analytical HPLC: 0% - 100% eluent B in 30min (gradient)
Capcell C8 (column)

Preparative HPLC: 0min – 15min: 20% eluent B (isocratic)
15min – 45min: 20% - 60% B (gradient)
Vydac Protein C4 (column)

Molecular Mass: 2451.6 Da (calculated average mass)
2460.6 Da (detected mass)

Bio-RLEELRETfeGlyLESLRKKLA-(S-Bzl) ($E_{K8TfeGly}$):

The peptide (0.1mmol) was synthesized on 4-sulfamylbutyryl resin, that was loaded with Fmoc-Ala-OH (0.65mmol/g). *(S)*-4,4,4-trifluoroethylglycine was synthesized in the group of Prof. Beate Koksch, according to a published strategy[235] and Fmoc-protected with Fmoc-Cl, applying a standard protocol. The non-natural amino acid was coupled twice for 12h and 19h, respectively, in 7mL DMF with 3 equivalents of each Fmoc-DfeGly-OH, HOAt, and DIC in both coupling steps. Fmoc-Glu(OtBu)-OH in amino acid position 7 was coupled twice for 12h and 8,5h, respectively, in 7mL DMF with 3 equivalents of each amino acid, HOAt, and DIC. Prior to Fmoc-deprotection of Glu7, free α-amino groups were capped. For purification, peptide was solved in 0.25% TFA / 50% ACN / 49.75% H_2O.

Analytical HPLC: 0% - 100% eluent B in 30min (gradient)
Vydac Protein C4 (column)

Preparative HPLC: 0min – 15min: 25% eluent B (isocratic)
15min – 45min: 25% - 60% B (gradient)
Vydac Protein C4 (column)

Molecular Mass: 2454.6 Da (calculated average mass)
2456.2 Da (detected mass)

Bio-RLEELREKTfeGlyESLRKKLA-(S-Bzl) ($E_{L9TfeGly}$):

The peptide (0.1mmol) was synthesized on 4-sulfamylbutyryl resin, that was loaded with Fmoc-Ala-OH (0.65mmol/g). *(S)*-4,4,4-trifluoroethylglycine was synthesized in the group of Prof. Beate Koksch, according to a published strategy and Fmoc-protected with Fmoc-Cl, applying a standard protocol. The non-natural amino acid was coupled twice for 12h and 19h, respectively, in 7mL DMF with 3 equivalents of each Fmoc-DfeGly-OH, HOAt, and DIC in both coupling steps. Fmoc-Lys(Boc)-OH in amino acid position 8 was coupled twice for 12h and 8,5h, respectively, in 7mL DMF with 3 equivalents of each amino acid, HOAt, and DIC. Prior to Fmoc-deprotection of Lys8, free α-amino groups were capped. For purification, peptide was solved in 0.25% TFA / 50% ACN / 49.75% H_2O.

Analytical HPLC: 0% - 100% eluent B in 30min (gradient)
Vydac Protein C4 (column)

Preparative HPLC: 0min – 15min: 20% eluent B (isocratic)
15min – 45min: 20% - 70% B (gradient)
Vydac Protein C4 (column)

Molecular Mass: 2469.6 Da (calculated average mass)
2471.4 Da (detected mass)

Bio-RLEELREDfpGlyLESLRKKLA-(S-Bzl) ($E_{K8DfpGly}$):

Peptide synthesis was content of a diploma thesis in the group of Prof. Beate Koksch. *(S)*-4,4-difluoropropylglycine was synthesized in the group of Prof. Beate Koksch, according to a published strategy[236] and Fmoc-protected with Fmoc-Cl, applying a standard protocol.

Bio-RLEELREKDfpGlyESLRKKLA-(S-Bzl) ($E_{L9DfpGly}$):

Peptide synthesis was content of a diploma thesis in the group of Prof. Beate Koksch. *(S)*-4,4-difluoropropylglycine was synthesized in the group of Prof. Beate Koksch, according to a published strategy and Fmoc-protected with Fmoc-Cl, applying a standard protocol.

Bio-RLEELREAibLESLRKKLA-(S-Bzl) (E_{K8Aib}):

The peptide (0.1mmol) was synthesized on 4-sulfamylbutyryl resin, that was loaded with Fmoc-Ala-OH (0.5mmol/g). Fmoc-protected aminoisobutyric acid (Aib) was purchased from Bachem. For coupling of this $C^{\alpha,\alpha}$-dialkylated amino acid to Leu9 of the peptide chain, 4 equivalents of each amino acid and HOAt were solved in 5mL DCM, applying ultrasonic. DMF was added in drops until HOAt was completely solved. After addition of 4 equivalents DIC, preactivation of Aib proceeded for 10min. Coupling reaction started with transfer of the preactivation solution to the resin and addition of 5mL DMF. The reaction proceeded for 30min. The procedure was repeated once. The same protocol was used for the coupling of Glu7 to Aib8. For purification, peptide was solved in 20% ACN / 80% H_2O.

Analytical HPLC: 0% - 100% eluent B in 30min (gradient)
Vydac Protein C4 (column)

Preparative HPLC: 0min – 15min: 20% eluent B (isocratic)
15min – 45min: 20% - 60% B (gradient)
Vydac Protein C4 (column)

Molecular Mass: 2400.6 Da (calculated average mass)
2402.6 Da (detected mass)

Bio-RLEELREKAibESLRKKLA-(S-Bzl) (E_{L9Aib}):

The peptide (0.1mmol) was synthesized on 4-sulfamylbutyryl resin that was loaded with Fmoc-Ala-OH (0.5mmol/g). Fmoc-protected aminoisobutyric acid (Aib) was purchased from Bachem. For coupling of this $C^{\alpha,\alpha}$-dialkylated amino acid to Glu10 of the peptide chain, 4 equivalents of each amino acid and HOAt were solved in 5mL DCM applying ultrasonic. DMF was added in drops until HOAt was completely solved. After addition of 4 equivalents DIC, preactivation of Aib proceeded for 10min. Coupling reaction started with transfer of the preactivation solution to the resin and addition of 5mL DMF. The reaction proceeded for 30min. The procedure was repeated once. The same protocol was used for the coupling of Lys8 to Aib9. For purification, peptide was solved in 20% ACN / 80% H_2O.

Analytical HPLC: 0% - 100% eluent B in 30min (gradient)
Vydac Protein C4 (column)

Preparative HPLC: 0min – 15min: 20% eluent B (isocratic)
15min – 45min: 20% - 60% B (gradient)
Vydac Protein C4 (column)

Molecular Mass: 2415.6 Da (calculated average mass)
2417.6 Da (detected mass)

Bio-RLEELREKMeLeuESLRKKLA-(S-Bzl) ($E_{L9MeLeu}$):

The peptide (0.1mmol) was synthesized on 4-sulfamylbutyryl resin, that was loaded with Fmoc-Ala-OH (0.65mmol/g). Fmoc-α-methylleucine (MeLeu) was purchased from Bachem. The couplings of MeLeu to Glu10 and Lys to MeLeu9 of the peptide chain were performed twice with 4 equivalents of amino acid, 4 equivalents PyBOP, 4 equivalents HOBt, and 8 equivalents DIEA. Reactions proceeded for 60min. For purification, peptide was solved in 20% ACN / 80% H_2O.

Analytical HPLC: 0% - 100% eluent B in 30min (gradient)
Vydac Protein C4 (column)

Preparative HPLC: 0min – 15min: 20% eluent B (isocratic)
15min – 45min: 20% - 60% B (gradient)
Vydac Protein C4 (column)

Molecular Mass: 2457.6 Da (calculated average mass)
2458.8 Da (detected mass)

Bio-RLEELRE$\underline{\text{TfmAla(I)}}$LESLRKKLA-(S-Bzl) ($E_{K8TfmAla(I)}$):

The peptide (0.1mmol) was synthesized on 4-sulfamylbutyryl resin, that was loaded with Fmoc-Ala-OH (0.65mmol/g). The racemic Z-protected TfmAla was synthesized in the group of Prof. Beate Koksch, according to a published strategy and incorporated into tripeptides. For the coupling of the tripeptide Fmoc-Ala-TfmAla(I)-Leu-OH to Glu10 of the peptide chain, 1.5 equivalents tripeptide and 1.6 equivalents HOAt were solved in 6mL DCM, applying ultrasonic. DMF was added in drops until HOAt was completely solved. The pH of the solution was adjusted to 7 – 7.5, using NMM. After addition of 1.6 equivalents DIC, preactivation of the tripeptide proceeded for 15min at 4°C. Coupling reaction started with transfer of the preactivated tripeptide to the resin and proceeded for 5h. The procedure was repeated with 1 equivalent tripeptide and 1.6 equivalents of each HOAt and DIC for 14h. The coupling of the following amino acid (Arg6) was performed 120min twice. For purification, peptide was solved in 0.25% TFA / 50% ACN / 49.75% H_2O.

Analytical HPLC: 0% - 100% eluent B in 30min (gradient)
Vydac Protein C4 (column)

Preparative HPLC: 0min – 15min: 25% eluent B (isocratic)
15min – 45min: 25% - 70% B (gradient)
Vydac Protein C4 (column)

Molecular Mass: 2396.6 Da (calculated average mass)
2398.7 Da (detected mass)

Bio-RLEELRETfmAla(II)LESLRKKLA-(S-Bzl) ($E_{K8TfmAla(II)}$):

The peptide (0.1mmol) was synthesized on 4-sulfamylbutyryl resin, that was loaded with Fmoc-Ala-OH (0.65mmol/g). The racemic Z-protected TfmAla was synthesized in the group of Prof. Beate Koksch, according to a published strategy and incorporated into tripeptides. For the coupling of the tripeptide Fmoc-Ala-TfmAla(II)-Leu-OH to Glu10 of the peptide chain, the same procedure like that of the coupling of Fmoc-Ala-TfmAla(I)-Leu-OH in $E_{K8TfmAla(I)}$ was applied. The coupling of the following amino acid (Arg6) was performed twice for 120min each coupling. For purification, peptide was solved in 0.25% TFA / 50% ACN / 49.75% H_2O.

Analytical HPLC: 0% - 100% eluent B in 30min (gradient)
Vydac Protein C4 (column)

Preparative HPLC: 0min – 15min: 25% eluent B (isocratic)
15min – 45min: 25% - 70% B (gradient)
Vydac Protein C4 (column)

Molecular Mass: 2396.6 Da (calculated average mass)
2398.7 Da (detected mass)

Bio-RLEELRERTfmAla(I)ESLRKKLA-(S-Bzl) ($E_{L9TfmAla(I)}$):

The peptide (0.1mmol) was synthesized on 4-sulfamylbutyryl resin, that was loaded with Fmoc-Ala-OH (0.53mmol/g). The racemic Z-protected TfmAla was synthesized in the group of Prof. Beate Koksch, according to a published strategy and incorporated into tripeptides. For the coupling of the tripeptide Fmoc-Lys(Boc)-TfmAla(I)-Glu(OtBu)-OH to Ser11 of the peptide chain, 1.5 equivalents tripeptide and 1.6 equivalents HOAt were solved in 6mL DCM applying ultrasonic. DMF was added in drops until HOAt was completely solved. The pH of the solution was adjusted to 7 – 7.5, using NMM. After addition of 1.6 equivalents DIC, preactivation of the tripeptide proceeded for 15min. Coupling reaction started with transfer of the preactivated tripeptide to the resin and proceeded for 8h. The procedure was repeated with same amounts of tripeptide and coupling reagents, and reaction proceeded for 14h. Prior to Fmoc-deprotection of Lys8, free α-amino groups were capped. For purification, peptide was solved in 0.1% TFA / 20% ACN / 79.9% H_2O.

Analytical HPLC: 0% - 100% eluent B in 30min (gradient)
Vydac Protein C4 (column)

Three main products were identified via analytical HPLC. Mass spectrometry revealed correct molecular masses for two of the separated peptides, which was most probably caused by racemization of Glu10 during segment coupling (Section 5.2.4.1). The third isolated product showed the mass of the peptide fragment [S^{11}-A^{17}]-$E_{L9TfmAla(I)}$. Obviously, the tripeptide was not coupled to this peptide fraction, and synthesis terminated after capped residue position Ser11.

Preparative HPLC: 0min – 15min: 20% eluent B (isocratic)
15min – 45min: 20% - 55% B (gradient)
Vydac Protein C4 (column)

Molecular Mass: 2469.6 Da (calculated average mass)
2475.7 Da (detected mass, D-Glu7 diastereomer)
2475.2 Da (detected mass, L-Glu7 diastereomer)

Bio-RLEELRERTfmAla(II)ESLRKKLA-(S-Bzl) ($E_{L9TfmAla(II)}$):

The peptide (0.1mmol) was synthesized on 4-sulfamylbutyryl resin, that was loaded with Fmoc-Ala-OH (0.53mmol/g). The racemic Z-protected TfmAla was synthesized in the group of Prof. Beate Koksch, according to a published strategy and incorporated into tripeptides. For the coupling of the tripeptide Fmoc-Lys(Boc)-TfmAla(II)-Glu(OtBu)-OH to Ser11 of the peptide chain, the same procedure like that of the coupling of Fmoc-Lys(Boc)-TfmAla(I)-Glu(OtBu)-OH in $E_{L9TfmAla(I)}$ was applied. Prior to Fmoc-deprotection of Lys8, free α-amino groups were capped. For purification, peptide was solved in 0.1% TFA / 20% ACN / 79.9% H_2O.

Analytical HPLC: 0% - 100% eluent B in 30min (gradient)
Vydac Protein C4 (column)

Three main products were identified via analytical HPLC. Mass spectrometry revealed correct molecular masses for two of the separated peptides, which was most probably caused by racemization of Glu10 during segment coupling (Section 5.2.4.1). The third isolated product showed the mass of the peptide fragment [S^{11}-A^{17}]-$E_{L9TfmAla(I)}$. Obviously, the tripeptide was not coupled to this peptide fraction, and synthesis terminated after capped residue position Ser11.

Preparative HPLC: 0min – 15min: 20% eluent B (isocratic)
15min – 45min: 20% - 50% B (gradient)
Vydac Protein C4 (column)

Molecular Mass: 2469.6 Da (calculated average mass)
2476.5 Da (detected mass, D-Glu7 diastereomer)
2477.2 Da (detected mass, L-Glu7 diastereomer)

7.1.3 Investigations on Thermal Stability of *Coiled Coil* Peptides

Prior to thermostability measurements of the substituted *coiled coil* dimers (P_X), electrophilic peptide fragments (E_X) had to be ligated to the nucleophilic peptide fragment (N) to obtain full-length peptide strands. Only experimental procedures are described in this section that were applied for the evaluation of amino acids after optimization of the screening method.

7.1.3.1 Ligation of the *Coiled Coil* Fragments

Reactions were catalyzed by *coiled coil* formation except ligation of E_{L9E}, where hydrophobic core interactions were to weak for an effective catalysis. A special liagtion protocol had to be applied for this peptide variant.

General Ligation Procedure:

Native Ligation: 65µL of a 1mM solution of N in 0.5% TFA / 99.5% H_2O, 90µL of a 500mM phosphate buffer pH 7.0, and 5µL of a 12.5mM solution of TCEP in water (pH adjusted to 7.0) were mixed in 1mL glas vials. Reduction of disulfide-linked N-dimers was allowed to proceed for 15min under agitation (1000rpm). Ligation reactions started with addition of 65µL E_X-solution (1mM in 0.5% TFA / 99.5% H_2O) and proceeded for 3h at RT / 1000rpm agitation. Analytical HPLC was used for monitoring of the reactions. Concentrated HCl was added to the reaction mixtures in drops until ligation products were completely solved. Prior to purification, reaction mixtures were stored over night at -30°C.

Reaction monitoring and product purification: HPLC was performed on a TSP device using a C8 / 5µm column (Phenomenex or Capcell) and UV (220nm) as well as fluorescence (λ_{ex}=230nm / λ_{em}=310nm) detection with a separation gradient of 30% - 50% eluent B in 30min. For reaction monitoring, 5µL of reaction mixture were diluted in 95µL 5% TFA / 45%

H_2O / 50% ACN to stop ligation reactions, and 80µL of the final dilution were loaded on column. Ligation products were purified in two portions each. Separated HPLC fractions were pooled and acetonitrile was evaporated. Aqueous solutions were washed twice with 200µL diethylether to remove benzylmercaptan from peptide solutions. Traces of diethylether were evaporated from the aqueous phases. The resulting peptide solutions (approx. 1mL) were frozen in liquid nitrogen and lyophyllized.

Ligation of E_{L9E} with N:

Native ligation: 0.25µmol E_{L9E} (approx. 0.6mg) and 0.5µmol N (approx. 1.5mg) were solved in a solution of 6M guanidine hydrochloride and 500mM phosphate buffer pH 7.0. For reduction of disulfides in N-dimers, 20µL benzylmercaptan were added. The reaction progress was monitored via analytical HPLC. To accelerate native ligation, pH was raised to 7.5 – 8 after 6h of reaction. After 9h of reaction, 1.5g N was added to further accelerate ligation. Although reaction was not yet finished, ligation product was purified from the reaction mixture via analytical HPLC after 24h overall reaction time.

Reaction monitoring and product purification: HPLC was performed on a TSP device using a C8 / 5µm column (Phenomenex) and UV (220nm) as well as fluorescence (λ_{ex}=230nm / λ_{em}=310nm) detection with a separation gradient of 30% - 50% eluent B in 30min. For reaction monitoring, 1µL of reaction mixture was diluted in 99 µL 5% TFA / 45% H_2O / 50% ACN to stop ligation reactions, and 80 µL of the final dilution were loaded on column. Reaction progress was monitored after 0 / 2 / 3 / 6 / 8 and 24h of native ligation. Benzylmercaptan was removed by washing the reaction mixture with 100µL diethylether, and ligation product was purified in 3 fractions á 25µL. Separated HPLC fractions were pooled, and acetonitrile was evaporated. The aqueous solution was again washed with 500µL diethylether and traces of ether were evaporated from the aqueous phase. The resulting peptide solution (approx. 1mL) was frozen in liquid nitrogen and lyophyllized.

7.1.3.2 Thermal Stability Measurements

Sample preparation: Solid peptides were resuspended in 200µL 100mM phosphate buffer pH 7.4 and 3µL of a TCEP solution (25mM, pH 7.4) were added. Peptide concentrations were determined via analytical HPLC. Therefore, 5µL of homogeneous peptide suspension were diluted in 95µL 5% TFA / 45% H_2O / 50% ACN, and 80µL of the dilution was loaded on the column. *Coiled coil* peptides were completely solved under acidic conditions. HPLC was

performed on a TSP device, using a Phenomenex C8 / 5µm column and fluorescence detection (λ_{ex}=230nm / λ_{em}=310nm) with a separation gradient of 30% - 50% eluent B in 30min. Analytical HPLC of a 100µM solution of N under identical conditions (includes 1:20 dilution in 5% TFA / 45% H_2O / 50% ACN) served as reference for the calculation of P_X concentrations. Peptide concentrations were calculated via determination of peak areas (detection of 3 tyrosine residues in the ligation products P_X and the nucleophilic peptide fragment N). For thermal unfolding experiments, final peptide solutions containing 20µM ligation products, 100mM phosphate buffer pH 7.4, and guanidine hydrochloride in certain concentrations (2, 3, or 5M) were prepared.

Thermal unfolding: Prior to unfolding experiments with the *coiled coil* solutions, TCEP was added to a final concentration of 250µM and disulfide reduction was allowed to proceed for 15min. 250µL of the final solutions were heated from 20°C to 90°C in a cuvette of 0.1 cm path length (quartz glass with lid) with a slope of 3°C/min. *Coiled coil* unfolding was recorded via observing of the CD-signal at 222nm with a nitrogen flow of 6L/min, a step resolution of 0.1°C and a band with of 10nm. The background of the GdnHCl / buffer solution was recorded separately and substracted from the peptide melting profiles. The resulting curves were fitted via the following five parameter equation, using the software Origin / version 6.0.

$$y = y_0 + \frac{a}{\left[1 + e^{-\left(\frac{x - x_0}{b}\right)}\right]^c}$$

7.1.4 Self-Replication Experiments

Only experimental procedures are described in this section, that were applied for the evaluation of amino acids after optimization of the screening method. Standard reactions were performed in 200mM phosphate buffer pH 7.0. For the evaluation of the sensitivity of the replicase reaction towards destabilization of the *coiled coil* structure to a certain extent, self-replication experiments with different concentrations of guanidine hydrochloride were performed.

General reaction conditions: Reactions were performed in 1mL glass vials at 22°C and pH 7.0. Peptide stock solutions (1mM in 0.5% TFA / 99.5% H_2O) were used to reach final peptide concentrations of 100µM in the reaction mixtures. Reactions were started by addition

of the electrophilic peptide fragments E_X. Prior to reaction start, TCEP was added to a final concentration of 250µM and reduction of disulfide-linked N-dimers was allowed to proceed for 15min. At definite times 10µL of reaction mixtures were diluted in 90µL 5% TFA / 45% H_2O / 50% ACN to stop ligation reaction, and reaction progress was monitored via analytical HPLC. Therefor, 80µL of the diluted aliquots were loaded on a C8 or C18 column (5µm / Phenomenex or Capcell) and HPLC was performed using fluorescence detection (λ_{ex}=230nm / λ_{em}=310nm) with a separation gradient of 30% - 50% eluent B in 30min. Turnover values were calculated from peak areas using the following equation (A: peak area; P: product; I: intermediate; N: nucleophilic peptide fragment):

$$\text{Turnover} = A_P / (A_N + A_{IP} + A_P)$$

Standard reactions: Self-replication reactions without guanidine hydrochloride were performed in 150µL overall volume with 200mM phosphate buffer pH 7.0. Therefore, the following stock solutions were used: 500mM phosphate buffer and 2.5mM TCEP. Aliquots for reaction monitoring were taken at 0 / 0.5 / 1 / 1.5 / 2 / 5 / 10 / 15 / 30 / 60 / and 120 min.

Reactions with increasing GdnHCl concentrations (0 – 2M): Self-replication reactions with low concentrations of guanidine hydrochloride were performed in 80µL overall volume with 100mM phosphate buffer pH 7.0. Therefore, the following stock solutions were used: 500mM phosphate buffer, 8M GdnHCl pH 7.0, and 2.5mM TCEP. Aliquots for reaction monitoring were taken at 15 / 30 / 60 / and 120 min.

Reactions with 6M GdnHCl: Self-replication reactions with 6M guanidine hydrochloride were performed in 150µL overall volume. Therefore, the following stock solutions were used: 500mM phosphate buffer, 8M GdnHCl pH 7.0, and 25mM TCEP. The pH values were checked at the beginning of the reactions. Aliquots for reaction monitoring were taken at 0 /30 / 60 / 120min as well as after 3 / 4 / 5 / 10 and 20h.

7.2 Molecular Modeling

Molecular mechanic and dynamic calculations were performed on SGI work stations Octane2, Indy5000, or Indigo Impact10000 (Silicon Graphics Industries), using the CHARMm 23.1 force field of the software QUANTA2000 v00.1110. Throughout the modeling studies, Gasteiger-calculated charge distributions were used.[237,238] The first part of the studies comprised the construction of an *in silico* model polypeptide that represents the *coiled coil*

screening model, used for the evaluation of the interaction properties of fluorinated amino acids. Subsequently, amino acid side chain conformations were systematically varied to characterize salt bridge formations of residues Arg1, Glu4, Lys8, Glu29, Lys32, and Glu36.

Construction of the protein model: The PDB-file of the x-ray structure of hepatitis δ antigen, a tetrameric protein, containing two identical antiparallel *coiled coil* dimers (PDB-entry: 1A92), served as the starting point for the generation of a protein model. In a first step, the amino acid sequence of the PDB structure was modified and adapted to the primary structure of the screening model (Section 5.1.1). Therefore, the protein was truncated to obtain the *coiled coil* part of a single antiparallel helix dimer. Both strands were elongated and amino acid positions were modified to construct a *coiled coil* dimer that represents the correct amino acid sequence of the *in vitro* screening protein. In the following step the structure had to be geometry-optimized in order to obtain the optimum conformation of all amino acid side chains in the *coiled coil*.
Prior to energy minimizations, side chain torsion angles of the amino acids in **e** and **g** positions of the *coiled coil* pattern were set up and resulted in conformations that support interhelical salt bridges. Since calculations without any parameter constraints resulted in the unfolding of the *coiled coil* structure, optimization of the dimer was performed stepwise, using constrained backbone torsion angles. Therefore, calculations were performed with a dielectric constant ε=80, applying the Adopted Basis Newton Raphson algorythm (ABNR) until the energy minimum was reached. After three optimization procedures (first with constraints, second without, and third again with constraints), values for torsion angle constraints were adapted to the newly generated structure and geometry optimization was repeated with constrained backbone torsion angles. After updating the constraint values, molecular dynamic studies were performed with the following parameters: 2ps heating to 300K, followed by a 2ps equilibration time period and 5ps simulation. The obtained average structure was energy minimized, using backbone torsion angle constraints and parameters of the former geometry optimizations. After final updating of the constraint values, the construction of an optimal *in silico* model for the *coiled coil* dimer was achieved.

Evaluation of salt bridge conformations: In further geometry optimization procedures, backbone torsion angles in a perimeter of 7Å around Lys8 and Glu36 were harmonically constrained (constraint value = 100.00), while all atom geometries outside this sphere were fixed (constraint value = -1). No constraints were set up for amino acid side chains within the radius of 7Å around the charged residues. Side chain torsion angles of the charged residues that may influence the K8-E29 salt bridge formation were altered systematically, following a rational strategy, and all reasonable conformations of the following salt bridge combinations

were set up and geometry optimized: R1-E4, R1-E36, E4-K8, K8-E29, K8-E36, E29-K32, and K32-E36. For all optimizations, a dielectric constant ε=80 was used and the first 10000 minimization steps were performed, applying the steepest descent algorythm (SD). ABNR was used subsequently until energy minimum was reached.

The geometries of the calculated salt bridges with the lowest energies were merged in new *coiled coil* structures. Thereby, all reasonable combinations were generated. Geometry optimizations were performed, using unchanged parameters with exception of the perimeter of harmonic backbone constraints and free side chains around Lys8 and Glu36, which was shifted to 9Å. Energies of the optimized structures were compared and most stable salt bridge combinations described.

7.3 Molecular Biology and Phage Display

7.3.1 Declaration of Suppliers

Chemicals, Reagents, and Antibiotics:

ABTS (2,2'-azino-di-[3-ethyl-benzthiazoline-sulfonate], Roche Molecular Biochemicals #102946), **A**crylamide (EM Science #1150), **A**garose (Gibco-BRL #15510-027; Roth #2267.3), **A**mmonium sulfate (Sigma #A-3678), **A**PS (ammonium persulfate, Sigma #A9164), **B**acto agar (BBL Difco #214030), **B**iotin (Sigma #HB-4501), **B**romophenol Blue (Merck #8122), **B**SA (Sigma #A-7906), **B**SA maleimide-conjugated (Pierce #7716), **C**abenicillin (Sigma #C1389-5G) **C**alcium chloride (Merck #1.02382), **C**oomassie Brilliant Blue G250 (Serva #17524.01), **D**isodium hydrogen phosphate (Meck #1.06580.1000), **D**MSO (Dimethylsulfoxide, Merck #1.02950.0500), **d**NTP mix 2,5 mM (Amersham Pharmacia Biotech #27-2035-02), **D**TT (1,4-dithiothreitol, Gibco BRL #15508-013; Roth #6908.2), **E**DTA (Ethylenediaminetetraacetic acid, Roth #8043.2), **E**thanol (Roth #9065.1), **E**thanolamine (Fisher Scientific #M251-1), **G**dnHCl (guanidine hydrochloride, AppliChem #A3240,0500), **G**lucose D-(+)- (Roth #X997.2), **G**lycerol anhydrous (Fluka #49767), **G**lycine for electrophoresis (Sigma #G8898), **I**PTG (isopropyl-β-D-thiogalactoside, Fisher Scientific #BP1620-10), (**K**anamycin monosulfate (Sigma #K4000; Roth #T832.2), **M**agnesium chloride (hexahydrate, Merck # 1.05833.0250), **M**ercaptoethylamine (Pierce #20408ZZ), N,N'-**M**ethylene-bis-acrylamide (ICN Biomedicals #800175), **M**ilk powder non-fat / Blotto (BioRad #170-6404; Roth #T145.2), **M**OPS (3-[N-morpholino] propanesulfonic acid, Sigma #M8899; Roth #6979.4), **O**range G (Merck #K30598525), **P**EG 8000 (polyethylene glycol, Sigma

#P5413/P2139), **P**eptone Tryptone (BD Biosciences #0123-17-3; Roth #8986.2), **P**NPP tablet (p-nitrophenyl phosphate, Sigma #N9389), **P**otassium chloride (Roth #6781.1), **P**otassium dihydrogen phosphate (Roth #3904.1), **P**rotease inhibitor Complete (Roche Applied Science #1836145), **S**DS (sodium dodecyl sulfate, AppliChem #A1112,0500), **S**odium acetate (Merck #1.06267), **S**odium azide (Fluka #71289), **S**odium chloride (Roth #3957.1), **S**treptavidin (Prozyme #SA10001; Roche Molecular Biochemicals #973190), **T**EMED (N,N,N',N'-tetramethylethylenediamine, ICN Biomedicals #805615), **T**ris (AppliChem #A1086,1000), **T**ween 20 (Sigma #P1379), **X**ylene cyanol (Sigma #X-2751), **Y**east extract (BD Biosciences # 0123-17-9; AppliChem #A1552,1000).

Antibodies and Enzymes:

Anti-FLAG BioM2 monoclonal antibody / biotinylated (Sigma #F 9291), **A**nti-M13 HRP-conjugated monoclonal antibody (Amersham Pharmacia Biotech #27-9421-01), **A**nti-M13 monoclonal antibody (Amersham Pharmacia Biotech #27-9410-01), **A**nti-M13 pIII monoclonal antibody (mouse, New England Biolabs #E8033S), **A**nti-MBP monoclonal antibody (Sigma #M-6295), **A**nti-mouse AP-conjugated antibody (IgG goat, Sigma #A-1047), **A**nti-mouse HRP-conjugated antibody (IgG goat, Sigma #A4416), **E**nterokinase light chain (New England Biolabs #P8070S), **E**xpand High Fidelity PCR System / DNA polymerase (Roche Applied Science #1732641), **H**ind III / restriction endonuclease (New England Biolabs #R0104S), **H**RP maleimide-activated (Pierce #0031485), **H**RP streptavidin-conjugated (Zymax #43-8323), **S**ac I / restriction endonuclease (New England Biolabs #R0156S), **S**fi I / restriction endonuclease (Roche Applied Science #1288032), **T**4 DNA Ligase (Gibco-BRL #15224090; New England Biolabs #M0202S), **T**aq DNA Polymerase (Qiagen #201203), Trypsin (Becton Dickinson # 0152-13-1; TPCK-treated, Sigma #T-1426)

Bacteria, Phage, and Plasmids:

***E.** coli* K12 ER2537 (New England Biolabs #801-N), ***E.** coli* ER2738 (New England Biolabs #E4104S), ***E.** coli* XL1-blue (Stratagene #200228), **V**CSM13 helper phage (Stratagene #200251), **p**Comb3H phagemid vector (GenBank database accession number: AF268280, Barbas laboratory, TSRI), **p**C3MBP plasmid vector (Barbas laboratory, TSRI)

Materials and Apparatures:

Bleach (5.25% sodium hydrochlorite solution, Clorox Comp.), **C**entrifugal filter units 0.22µm (Millipore #UFC30GV00), **C**entrifuges: Sorvall RC-5B / 5C with rotors Sorvall GS-3 / GSA and SS-34 (Du Pont Instruments); table-top centrifuge Biofuge Pico (Hereus), **D**NA marker 2-log (New England Biolabs #N3200S), **D**NA marker 100bp (Amersham Pharmacia Biotech #27-4001-01), **D**NA marker 1kbp (Gibco-BRL #15615-024), **E**CL plus western blotting reagent (Amersham Pharmacia Bioscience #RPN2132), **E**LISA plate 96-well (Corning Costar #3690), **E**LISA plate 96-well lids (Corning Costar #3930), **E**LISA plate 96-well sealer adhesive back (ICN Biomedicals #76-401-05), **E**lutrap electro separation system (Schleicher & Schuell #10447724), **F**ilter Millex-GS sterilized unit 0.22µm (Millipore #SLGS025OS), **G**ene pulser apparature / Pulse Controller (BioRad; Equibio Easyject Prima), **M**ineral Oil for PCR (Sigma #M5904), **P**rotein marker ProSieve Color (Cambrex #50550), **P**VDF Hybond-P transfer membrane for western blot (Amersham Pharmacia Biosciences #RPN2020F); Millipore #ISEQ26260), **Streptavidin**-coated magnetic particles (Dynal Biotech M-280 #112.05; Roche Applied Science #11641778001), **Q**iafilter Plasmid Maxi Kit 25 (Qiagen #12263), **Q**iaprep Spin Miniprep 250 (Qiagen #27106), **Q**iaquick gel extraction kit (Qiagen #28706), **Q**iaquick PCR purification kit (Qiagen #28104)

7.3.2 Recipes

ABTS 50x: 1g ABTS was dissolved in deionized water (20mg/mL), aliquoted, and stored at -20°C.

Agar plates: Luria Broth (LB) agar was melted in a microwave oven and cooled down to approx. 50°C. For Carb-plates, carbenicillin stock solution was added to a final concentration of 100µg/mL and agar was poured into plate dishes (approx. 30mL/plate). Plates were stored at 4°C.

AP developer: 50mL diethanolamine (10%), 525µL 1M magnesium chloride (0.01%), and 98mg sodium azide (3mM) were dissolved in deionized water and brought to a final volume of 500mL. After adjustment of the pH to 9.8 with 12N HCL, solution was filter-sterilized (0.22µm) and stored in the dark.

Carbenicillin stock solution: Carbenicillin was dissolved in distilled water (100mg/ml), filter-sterilized (0.22µm), aliquoted, and stored at -20°C.

Citrate buffer 10x: 6.62g citric acid and 5.44g sodium citrate were dissolved in deionized water, pH was adjusted to 4.0, and solution was brought to a final volume of 100mL. Buffer was filter-sterilized (0.22µm) and stored at RT.

Coomassie staining solution: 0.2g Coomassie Brilliant Blue G250 were dissolved in 25mL Ethanol, 17mL perchloric acid and 400mL deionized water. The staining solution was stored at RT.

DNA Gel Loading dye 5x: 3.45mL glycerol (30%), 186.2mg EDTA (50mM) and 10mg dye (0.1%) were dissolved in deionized water and brought to a final volume of 10mL. The following dyes were used as color markers for agarose gel electrophoresis: orange G (200bp), bromophenol blue (500bp), and xylene cyanol (2kbp). Mixtures of the loading buffers were applied for electrophoreses.

DTT stock solution (1M): 1.55g DTT were dissolved in 0.01M sodium acetate (pH 5.2) and brought to a final volume of 10mL. Solution was filter-sterilized (0.22µm), aliquoted, and stored at -20°C.

Electroblotting buffer: 3g Tris base, 14.4g glycol, and 100mL methanol were dissolved in deionized water and brought to a final volume of 1L. After filter sterilization, buffer was stored at RT.

Enterokinase light chain (EK_L) reaction buffer: 2.423g Tris (20mM), 2.922g sodium chloride (50mM), and 294mg calcium chloride (2mM) were dissolved in deionized water and brought to a final volume of 1L. After adjustment of pH to 7.4, buffer was autoclaved and stored at RT.

Enterokinase light chain (EK_L) storage buffer: 61mg Tris (20mM), 292mg sodium chloride (200mM), and 7.4 mg calcium chloride were dissolved in deionized water and 29mL of 87% glycerol (50%) were added. The solution was brought to a final volume of 50mL and pH was adjusted to 7.2. After autoclaving buffer was stored at RT.

HRP developer (for one 96-well ELISA plate): 120µL 50x ABTS, 1.8µL 30% H_2O_2, and 0.6mL 10x citrate buffer were dissolved in deionized water and brought to a final volume of 6mL. The developer was prepared shortly before use.

IPTG stock solution: 2.38 g IPTG were dissolved in deionized water, brought to a final volume of 10mL, sterilized (0.22µm), aliquoted, and stored at -20°C.

Kanamycin stock solution: Kanamycin was dissolved in distilled water (50mg/mL), filter-sterilized (0.22µm), aliquoted, and stored at -20°C.

Luria Broth (LB) agar: 10g tryptone peptone, 5g yeast extract, 5g sodium chloride, and 16g bacto agar were dissolved in deionized water and brought to a final volume of 1L and divided into aliquots. Agar was autoclaved and stored at RT.

Luria Broth (LB) medium: 10g tryptone peptone, 5g yeast extract, and 5g sodium chloride were dissolved in deionized water and brought to a final volume of 1L. After adjustment of pH with NaOH (7.0) medium was autoclaved and stored at 4°C.

Luria Broth (LB) top agar: 625mg tryptone peptone, 312.5 mg yeast extract, 312.5 mg sodium chloride, and 358mg bacto agar were dissolved in deionized water and bought to a final volume of 50mL. Agar was autoclaved and stored at 4°C.

PBS-T: Phosphate Buffered Saline (PBS) containing 0.1% (v/v) Tween 20

PEG/NaCl solution: 100g polyethylene glycol-8000 (20%) and 73g sodium chloride (2.5M) were dissolved in deionized water and brought to a final volume of 500mL and stored at 4°C.

Phosphate Buffered Saline (PBS): 8.0g sodium chloride, 0.2g potassium chloride, 1.7g disodium hydrogen phosphate, and 0.163g potassium dihydrogen phosphate were dissolved in deionized water and brought to a final volume of 1L. After adjustment of pH to 7.4 with HCl, saline was autoclaved and stored at RT.

SDS-loading buffer: 1.2mg bromophenol blue were dissolved in 12mL 10% SDS, 6mL glycerol, 1mL 1 M Tris-HCl pH 6.8, and 10mL deionized water. Buffer was stored at RT.

SDS-PAGE buffer: 3.03g Tris base, 14.4g glycine, and 1g SDS were dissolved in deionized water and brought to a final volume of 1L. After filter sterilization, the buffer was stored at RT.

SOC medium: 4g tryptone peptone, 1g yeast extract, and 0.1g sodium chloride were dissolved in 150mL deionized water. After addition of 2mL 0.25M potassium chloride and 2mL 1M magnesium chloride, pH was adjusted to 7.0 and the solution was brought to a final

volume of 200mL. Medium was divided into 50mL portions and autoclaved. Finally, 200µL 1M glucose solution (filter-sterilized; 0.22µm) were added. Stock solution was stored at 4°C.

Sodium acetate 3M pH 5.2: 16.33g sodium acetate were solved in deionized water and brought to a final volume of 40mL. After pH adjustment with acetic acid solution was autoclaved and stored at RT.

Streptavidin stock solution: 10mg streptavidin was dissolved in 5mL deionized water (2mg/mL), filtersterilized, aliquoted and stored at -20°C.

Super Broth (SB) medium: 10g MOPS, 20g yeast extract, and 30g tryptone peptone were dissolved in deionized water and brought to a final volume of 1L. After pH adjustment to 7.0 the medium was autoclaved and stored at 4°C.

TAE buffer 100x: 484g Tris base were solved in 200mL deionized water. After addition of 114.2mL acetic acid and 200mL 0.5M EDTA pH 8.0, the buffer was brought to a final volume of 1L and filter-sterilized (0.22µm).

TBS-T: Tris Buffered Saline (TBS), containing 0.1% (v/v) Tween 20

TE buffer pH 7.4: 500mM Na_2EDTA pH 8.0 was added to 10mM Tris buffer pH 7.4 to reach a final concentration of 1mM. Buffer was autoclaved and stored at RT.

Tris Buffered Saline (TBS): 8.7g sodium chloride and 6.1g Tris base were dissolved in deionized water and brought to a final volume of 1L. After adjustment of pH to 7.4 with HCl, saline was autoclaved and stored at RT.

7.3.3 General Techniques

DNA quantification after purification or precipitation was perfomed with spectrophotometers. DNA concentrations were calculated using the following formulas, while OD = optical density, DF = dilution factor, SF = specificity factor (50 for dsDNA, 33 for ssDNA, 20 for oligonucleotides), and d = path length in cm:

$c[ng/\mu L] = OD_{260} \times DF \times SF \times d^{-1}$

The purity of the DNA samples was determined via the OD_{260} / OD_{280} ratio. A value of 1.7 – 1.8 revealed a sufficient purity of the prepared DNA.

The following primers were used for PCR-colony scans and the sequencing of DNA inserts, that were fused to the 5'-end of geneIII in pComb3H phagemid:

ompseq (sense primer): 5'-AAG ACA GCT ATC GCG ATT GCA G-3'
gback (backward primer): 5'-GCC CCC TTA TTA GCG TTT GCC ATC-3'

7.3.3.1 Plasmid DNA Isolation from Cell Cultures

Plasmid DNA was isolated from *E. coli* cell cultures and purified, using plasmid DNA preparation kits (Qiagen). Depending on how much DNA was needed, plasmid mini- or maxipreparations were performed.

Plasmid Minipreparation: A bacteria single clone colony from a carbenicillin plate dish was inocculated in 3mL prewarmed SB medium containing 67µg/mL carbenicillin, and culture was allowed to grow for 16h at 37°C and 200rpm agitation. Bacteria were spinned down using table-top centrifuges either in 15mL falcon tubes for 6min at 3500rpm or in 1.5mL centrifuge tubes (2 fractions per sample) for 1min at 13000rpm. Supernatant was discarded, and plasmid DNA was isolated and purified using Qiaprep Spin Miniprep 250 kit, according to the protocol provided by the supplier. Finally, DNA was eluted from the column, using 25µL deionized water or 50µL 10mM Tris buffer pH 8.5 and stored at -20°C. DNA concentrations of 300 – 600ng/µL have usually been obtained.

Plasmid Maxipreparation: A bacteria single clone colony from a carbenicillin plate dish was inocculated in 5mL prewarmed SB medium containing 500µg/mL carbenicillin, and culture was allowed to grow for 8h at 37°C and 200rpm agitation. Cell culture was transferred to 95mL prewarmed SB medium containing 500µg/mL carbenicillin and was allowed to grow for 16h at 37°C and 200rpm agitation. Plasmid DNA was isolated and purified, using Qiafilter Plasmid Maxi Kit 25, according to the protocol provided by the supplier. Finally, DNA pellets were dissolved in 150µL TE buffer pH 7.4 and stored at -20°C. DNA concentrations of 3000 – 4500ng/µL have usually been obtained.

7.3.3.2 DNA Preparation and Purification

DNA was separated via agarose gelelectrophoresis, using an appropriate percentage of agarose and voltage, respectively, or by applying a Qiaquick PCR purification kit (for work-up of DNA from PCR reactions). DNA was isolated from agarose gels either via centrifugation of centrifugal filter units or by applying a Qiaquick gel extraction kit. Ethanol precipitation was performed to precipitate and dry DNA. If highly pure opened plasmid DNA was needed (library construction), purification via agarose gel electrophoresis and subsequent electroelution was performed.

Agarose gel electrophoresis: DNA solutions were mixed with 0.25 volumes of 5x DNA gel loading dye and loaded on an agarose gel, containing 20ng/mL ethidium bromide. If not otherwise declared, *stem loop*-encoding DNA fragments and PCR colony scan products were analyzed on 2% agarose gels, opened plasmid DNA on 0.7% gels. Electrophoreses were performed at 80-100V.

Ethanol precipitation of DNA: After determination of the volume of the DNA solution, 2.5 volumes ethanol and 0.1 volume 3M sodium acetate pH 5.2 were added. DNA was allowed to precipitate for 30min at -80°C or over night at -20°C. After centrifugation for 15min at 13000rpm and 4°C in a table-top centrifuge, supernatant was discarded, and DNA was allowed to dry on air for not longer than 3min. DNA was dissolved in deionized water or appropriate buffers, depending on subsequent applications.

Isolation from agarose gels using centrifugal filter units: Agarose gel slices were applied onto a filter unit and centrifuged for 15min at 7000rpm and 4°C using a table-top centrifuge. DNA was isolated via ethanol precipitation and the concentration was determined.

DNA purification via agarose gelelectrophoresis and Electroelution: 100µg circular plasmid DNA were digested with restriction endonucleases, according to protocols suggested for the respective enzymes and separated on 0.7% agarose gels. The plasmid bands were cut out and DNA was purified via electroelution, applying an Elutrap device. Electroelution was performed over night at 100V, according to the protocol provided by the supplier. DNA was isolated via ethanol precipitation and concentration was determined.

7.3.3.3 PCR-Colony Scan and DNA Sequencing

To evaluate if pComb3H-transfected bacteria clones possess the DNA insert of right size, which is fused to the 5'-end of geneIII, test PCRs with bacteria colonies were performed. Therefor, trace amounts of single bacteria colonies were picked from plate dishes and mixed with the PCR solution. A phagemid preparation with an insert of the right size served as a positive control, a blank sample without DNA or bacteria as negative control.

PCR sample:

5µL 10x PCR buffer
5µL 2.5mM dNTP mix
0.5µL ompseq (250ng/µL)
0.5µL gback (250ng/µL)
0.25µL Taq-polymerase
38.75µL deionized water

PCR program:

94°C for 2min
94°C for 0.5min ⎫
55°C for 0.5min ⎬ 25 cycles
72°C for 0.5min ⎭
72°C for 10min
4°C until utilization

PCR products were analyzed via agarose gel electrophoresis. Bands between 400 and 500bp revealed inserts of the right size (Figure 7).

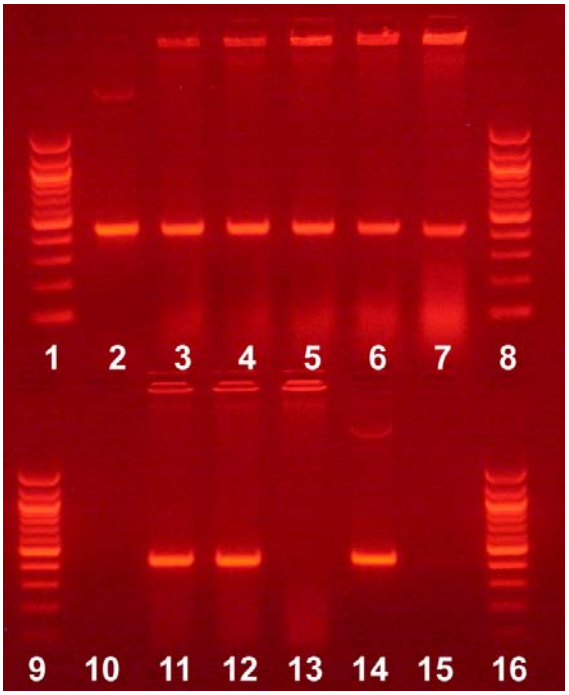


Figure 7.1: *Agarose gel picture of a sample PCR-colony scan. Lanes 1, 8, 9, 16: 2-log DNA marker (100bp steps bottom-up), lane 2: positive control, lanes 3-7 and 11-14: sample colonies, lane 15: negative control (lane 10: free).*

The picture shows that all tested sample clones with exception of that in lane 13 possess the DNA insert of the right size.

Since a PCR-colony scan can only prove the size of the DNA insert, the correct sequence had to be determined via DNA sequencing.

For DNA sequencing, plasmid minipreparations of pComb3H were provided. Sequencing was performed by the core facility of the Scripps Research Institute (sample preparation: 2µg plasmid DNA in 10µL deionized water) or by MWG-Biotech (sample preparation: 2µg plasmid DNA in 20µL 5mM Tris-HCl, pH 8.5) using primers ompseq or gback.

7.3.4 Preparation of Electrocompetent Cells

***E. coli* ER2537 (genotype):** λ- F' *lacI^q Δ(lacZ)M15 proA$^+$B$^+$ /fhuA2 supE Δ(lac-proAB) thi Δ(hsdMS-mcrB)5* (r_K^- m_K^- McrBC$^-$)

***E. coli* K12 ER2738 (genotype):** F'*proA$^+$B$^+$ lacI^q Δ(lacZ)M15 zzf::Tn*10(Tet^R)*/ fhuA2 supE glnV Δ(lac-proAB) thi-1*

***E. coli* XL1-blue:** *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB lacI*q*ZΔM15* Tn*10* (Tetr)]

Electrocompetent *E. coli* XL1-blue and ER2537 were taken from stocks in the laboratory of Prof. Barbas at TSRI. Electrocompetent *E. coli* ER2738 were prepared, following the procedure described below. All materials and liquids were autoclaved prior to use. Cell resuspension was performed using 5mL pipette tips with capped ends.

Cell growth: Commercially purchased ER2738 were grown on a plain agar plate dish over night at 37°C. 100mL prewarmed LB medium in a 250mL Erlenmeyer flask were inocculated with fresh bacteria from the plate, and cell culture was allowed to grow for 16h at 37°C and 200rpm agitation. 2 x 0.5mL of this culture were transferred to 2 x 500mL prewarmed SB medium in 2L Erlenmeyer flasks and were allowed to grow at 37°C and 200rpm agitation until OD_{595} reached 0.5 (approx. 3h). Cell culture was divided into aliquots and transferred to centrifuge bottles, that were precooled on ice. Cells were centrifuged for 10min at 3000g (4000rpm, using a GSA rotor) / 4°C and supernatant was discarded. The following washing procedure was performed on ice.

Washing of log-phase cells: All six cell pellets were resuspended in 5mL deionized water (precooled on ice), pooled and allocated to two centrifuge bottles. Bottles were filled up with cold deionized water until fill level of approx. 75% was reached. Bacteria were centrifuged for 10min at 3000g (4000rpm, using a GSA rotor) / 4°C and supernatants were discarded. The washing of the cells with deionized water was repeated once. Cell pellets were resuspended

with 5mL precooled 10% glycerol and transferred to centrifuge tubes with conical bottoms. Tubes were filled up with 10% glycerol to a final volume of 20mL and centrifuged for 10min at 3000g (5000rpm, using rotor SS-34). Supernatants were discarded, and washing with 10% glycerol was repeated once. After washing, cells were aliquoted.

Aliquoting of electrocompetent cells: Cell pellets were resuspended in 1mL 10% glycerol each and pooled. The resulting suspension was diluted with 10% glycerol to obtain OD_{595} = 1 (measuring 1/100 dilutions). The cell suspension was divided into 50µL aliquots, transferred to 0.6 mL centrifuge tubes (precooled at -80 °C), and immediately frozen in liquid nitrogen. Aliquots were stored at -80°C.

7.3.5 Molecular Cloning

For molecular cloning of the different *stem loop* constructs into pComb3H and pC3MBP, the DNA fragments encoding for the protein had to be designed and prepared. After digest with Sfi I, ligation to the 5'-end of geneIII on the respective vector was performed, and *E. coli* was transfected with the vector. Single bacteria clones were selected and *stem loop*-encoding DNA was analyzed via PCR colony scan and DNA sequencing. Plasmid DNA of a clone having the right DNA sequence was then enriched and purified via plasmid DNA preparation from *E. coli* cell cultures.

7.3.5.1 Construction of *Stem Loop*-Encoding DNA Fragments

DNA fragments have either been constructed *ab initio* via overlap extension PCR from 4 oligonucleotides or via PCR mutagenesis of previously constructed *stem loop*-encoding DNA fragments. For some of the DNA constructs, different PCR strategies had to be tested in order to obtain clones of the right DNA sequence. Only experimental protocols that led to correct DNA fragments will be described in this section.

Construction of (t)-and (o)-encoding DNA (Section 5.3.1 / Figures 5.4 and 5.24):

Two mutants of the DNA fragment that encodes for the *stem loop* fragment of the original design were constructed: one encoding for the whole protein fragment (t) and another possessing two stop codons in amino acid positions Glu29 and Leu30 (o). DNA fragments were prepared by overlap extension PCR, applying a one-step strategy. Therefore, the following oligonucleotides were synthesized in the laboratory of Prof. Barbas at TSRI and

used as primers (codons are in reading frame; overlapping parts are shaded in grey, Sfi I sites are shown in red and blue, Hind III sites in yellow, and Sac I sites in green):

forward primer I: 5'-TTC GCT ACC GTG GCC CAG GCG GCC TGC CTG AAA CGT GAA CTG CGT AAA CTG GAA AAA GAA CTG AAG AAA CTG GAA GAA GAA CTG-3'

backward primer I: 5'-CTT CTC GCG CAG CTC CTC AAG GCG TGG ACC ACC ACC CGG TTC CAG AGA AGA CAG TTC TTC TTC CAG TTT CTT CAG TTC TTT TTC-3'

forward primer II: 5'-GGT CCA CGC CTT GAG GAG CTG CGC GAG AAG CTG GAG TCT CTT CGC AAG AAA CTT GAG GAG CTT AAG CGC GAG CTG CGC AAG CTT GAG AAG GAG CTT-3'

backward primer II (t): 5'-GAG GTG ATG GTG CTG GCC GGC CTG GCC GAG CTC TTC GAG GGA GGA GAG CTC TTC CTC CCT CTT TTT AAG CTC CTT CTC AAG CTT GCG CAG CTC-3'

backward primer II (o): 5'-GAG GTG ATG GTG CTG GCC GGC CTG GCC GAG CTC TTC GAG GGA GGA GAG CTC TTC TCA TCA CTT TTT AAG CTC CTT CTC AAG CTT GCG CAG CTC-3'

The following PCRs were performed to obtain the full-length DNA constructs (t) and (o):

PCR sample:

10µL 10 x PCR buffer
10µL 2.5mM dNTP mix
1µL forward primer I (250ng/µL)
1µL backward primer I (250ng/µL)
1µL forward primer II (250ng/µL)
1µL backward primer II (250ng/µL)
0.5µL Taq-polymerase
75.5µL deionized water

PCR program:

94°C for 2min
94°C for 0,5min ⎫
55°C for 0.5min ⎬ 25 cycles
72°C for 0.5 min ⎭
72°C for 10min
4°C until utilization

The PCR products were purified via ethanol precipitation, dissolving in 40µL deionized water and subsequent agarose gel electrophoresis. DNA bands were cut out and DNA was isolated using centrifuge filter units and ethanol precipitation. After dissolving in 20µL deionized water, the DNA was stored at -20°C.

Construction of (nt)-and (no)-encoding DNA (Section 5.3.4.2 / Figure 5.33):

As described for the construction of (t) and (o) DNA, two mutants of the DNA fragment that encodes for the *stem loop* fragment with a redesigned charged interface were constructed: one encoding for the whole protein fragment (nt), and another possessing two stop codons in amino acid positions Glu29 and Leu30 (no). DNA fragments were prepared by overlap extension PCR, applying a one-step strategy with trace amounts of both middle primers and a polymerase with proof-reading activity. Therefore, the following oligonucleotides were synthesized in the laboratory of Prof. Barbas at TSRI and used as primers (codons are in reading frame; overlapping parts are shaded in grey, Sfi I sites are shown in red and blue, Hind III sites in yellow, and Sac I sites in green):

forward primer I: 5'-TTC GCT ACC GTG GCC CAG GCG GCC TGC CTG AAA CGT AAA CTG CGT AAA CTG AAG AAA AAG CTG AAG AAA CTG CGC GAA CGC CTG-3'

backward primer I: 5'-TTC CTC TTC CAG CTC CTC AAG TTC TGG ACC ACC ACC CGG GCG CAG AGA AGA CAG GCG TTC GCG CAG TTT CTT CAG CTT TTT CTT-3'

forward primer II: 5'-GGT CCA GAA CTT GAG GAG CTG GAA GAG GAA CTG GAG TCT CTT GAA AAG GAA CTT GAG GAG CTT GAA CGC GAG CTG CGC AAG CTT GAG AAG GAG CTT-3'

backward primer II (t): 5'-GAG GTG ATG GTG CTG GCC GGC CTG GCC GAG CTC TTC GAG GGA GGA GAG TTC TTC CTC AAG CTT TTT AAG CTC CTT CTC AAG CTT GCG CAG CTC-3'

backward primer II (o): 5'- GAG GTG ATG GTG CTG GCC GGC CTG GCC GAG CTC TTC GAG GGA GGA GAG TTC TTC TCA TCA CTT TTT AAG CTC CTT CTC AAG CTT GCG CAG CTC -3'

The PCRs described below were performed to obtain the full-length DNA constructs (t) and (o). Prior to this DNA construction, test PCR experiments with six different annealing temperatures (53-63°C) and with or without 5µL DMSO were performed to evaluate optimal PCR conditions. In these tests, 1µL of all primers (250ng/µL) were added to the reaction. All other conditions conform to the following protocol:

PCR sample:

10µL 10 x PCR buffer
10µL 2.5mM dNTP mix
4µL forward primer I (250ng/µL)
4µL backward primer II (250ng/µL)
2µL forward primer II (10ng/µL)
2µL backward primer I (10ng/µL)
0.5µL High-Fidelity-polymerase
67.5µL deionized water

PCR program:

94°C for 2min
94°C for 0.5min ⎫
58°C for 0.5min ⎬ 25 cycles
72°C for 0.5min ⎭
72°C for 10min
4°C until utilization

The PCR products were purified via agarose gel electrophoresis. DNA bands were cut out, and the DNA was isolated using centrifuge filter units and ethanol precipitation. After dissolving in 20µL deionized water, the DNA was stored at -20°C.

Construction of (nt-MBP)-and (no-MBP)-encoding DNA (Section 5.3.4.3):

Since the vectors pComb3H and pC3MBP possess identical cloning sites for the cloning of DNA that encodes for proteins to be displayed as N-terminal fusions of pIII and MBP, respectively, (nt)- and (no)-DNA fragments were cut out of pComb3H via Sfi I digest and ligated into opened pC3MBP. No new stem loop-encoding DNA had to be prepared.

Construction of (ft)-encoding DNA (Section 5.3.4.5 / Figure 5.38):

Only the mutant of the pIII-MBP-*stem loop*-encoding DNA without stop codons was prepared (ft). Therefore, a two-step PCR mutagenesis was applied, that used the (nt-MBP) clone of pC3MBP as template. The following oligonucleotides were synthesized in the laboratory of Prof. Barbas at TSRI and used as primers (new Sfi I site is shown in red):

forward primer I (ompseq): 5'- AAG ACA GCT ATC GCG ATT GCA G-3'

backward primer I: 5'-GAC CTG CTT GAC CGA GCT CTT CGA GGG AGG-3'

forward primer II: 5'-CGG TCA AGC AGG TCA GAT GAA AAT CGA AGA AGG-3'

backward primer II: 5'-GAG GTG ATG GTG CTG GCC GGC CTG GCC AGA GGA TCC AAA TTC TGA AAT CC-3'

In the first mutagenesis step, two PCRs were performed to produce both half of the new DNA fragment, having the Sfi I site between the *stem loop* and MBP genes mutated and a new Sfi I site at the 3'-end of the MBP gene generated. PCR 1a produced the fragment upstream from the mutated Sfi I (forward primer I / backward primer I) site and PCR 1b produced the fragment downstream from the mutated cloning site (forward primer II / backward primer II):

PCR sample:
10µL 10 x PCR buffer
10µL 2.5mM dNTP mix
1µL template (nt-MBP / 100ng/µL)
4µL forward primer I / II (250ng/µL)
4µL backward primer I / II (250ng/µL)
0.5µL High-Fidelity-polymerase
70.5µL deionized water

PCR program:
94°C for 2min
94°C for 0.5min ⎫
57°C for 0.5min ⎬ 17 cycles
72°C for 0.5min ⎭
72°C for 10min
4°C until utilization

The PCR products were purified via agarose gel electrophoresis on a 1.5% agarose gel. DNA bands were cut out, and DNA was isolated using centrifuge filter units and ethanol precipitation. After dissolving in 30µL deionized water and determination of DNA concentration, the DNA was stored at -20°C.

In the second mutagenesis step (PCR 2) the products of PCRs 1a and 1b were elongated and amplified to obtain the full-length (ft)-DNA fragment.

PCR sample:
10µL 10 x PCR buffer
10µL 2.5mM dNTP mix
1.5µL template I (PCR 1a / 74ng/µL)
2µL template II (PCR 1b / 51ng/µL)
4µL forward primer I (250ng/µL)
4µL backward primer II (250ng/µL)
0.5µL High-Fidelity-polymerase
68µL deionized water

PCR program:
94°C for 2min
94°C for 1min ⎫
57°C for 0.5min ⎬ 17 cycles
72°C for 3min ⎭
72°C for 10min
4°C until utilization

The PCR products were purified via agarose gel electrophoresis on a 1% agarose gel. DNA bands were cut out, and DNA was isolated using centrifuge filter units and ethanol precipitation. After dissolving in 30µL deionized water, the DNA was stored at -20°C.

Construction of (at)-encoding DNA (Section 5.3.4.6):

The (at)-DNA construct was similar to the (nt) analogue with the exception of one codon. Compared to (nt), the TGA codon adjacent to the 3'-end of the 5'-Sfi I site was mutated to GCA (Cys→Ala mutation). The DNA fragment was prepared by overlap extension PCR. Therefore, forward primer I was synthesized at Free University Berlin (codons are in reading frame; overlapping part is shaded in grey, Sfi I site is shown in blue):

forward primer I: 5'-TTC GCT ACC GTG GCC CAG GCG GCC TGC CTG AAA CGT AAA CTG CGT AAA CTG AAG AAA AAG CTG AAG AAA CTG CGC GAA CGC CTG-3'

Forward primer II and backward primers I and II (t) were adopted from the (nt)-DNA fragment construction PCR.

PCR sample:
10µL 10 x PCR buffer
10µL 2.5mM dNTP mix
14µL forward primer I (75ng/µL)
4µL backward primer II (250ng/µL)
2µL forward primer II (10ng/µL)
2µL backward primer I (10ng/µL)
0.5µL High-Fidelity-polymerase
75.5µL deionized water

PCR program:
94°C for 2min
94°C for 0.5min ⎫
58°C for 0.5min ⎬ 25 cycles
72°C for 1min ⎭
72°C for 10min
4°C until utilization

The PCR products were purified via agarose gel electrophoresis. DNA bands were cut out, and DNA was isolated using a Qiaquick gel extraction kit. After elution from the column with 30µL deionized water, the DNA concentration was determined and the DNA was stored at -20°C.

Construction of (et)-encoding DNA (Section 5.3.4.7 / Figure 5.43):

The (et)-encoding DNA fragment was prepared by PCR mutagenesis using the (nt)-phagemid clone as template. Two oligonucleotides were synthesized at Free University Berlin and used as PCR primers. The forward primer possessed the enterokinase recognition site within the overhang (codons are in reading frame; template binding fractions are shaded in grey).

forward primer: 5'-TTC GCT ACC GTG GCC CAG GCG GCC GAC TAC AAA GAC GAC GAC GAC AAA TGC CTG AAA CGT AAA CTG CGT AAA CTG AAG AAA AAG-3'

backward primer: 5'-GAG GTG ATG GTG CTG GCC GGC-3'

PCR sample:

10µL 10 x PCR buffer
10µL 2.5mM dNTP mix
10µL template (nt / 10ng/µL)
4µL forward primer I (250ng/µL)
4µL backward primer I (250ng/µL)
0.5µL High-Fidelity-polymerase
61µL deionized water

PCR program:

94°C for 2min
94°C for 0.5min ⎫
56°C for 0.5min ⎬ 28 cycles
72°C for 1min ⎭
72°C for 10min
4°C until utilization

The PCR products were purified via agarose gelelectrophoresis. DNA bands were cut out, and DNA was isolated using a Qiaquick gel extraction kit. After elution from the column with 30µL 10mM Tris buffer pH 8.5, DNA concentration was determined, and the DNA was stored at -20°C.

7.3.5.2 Sfi I Digest of PCR Products

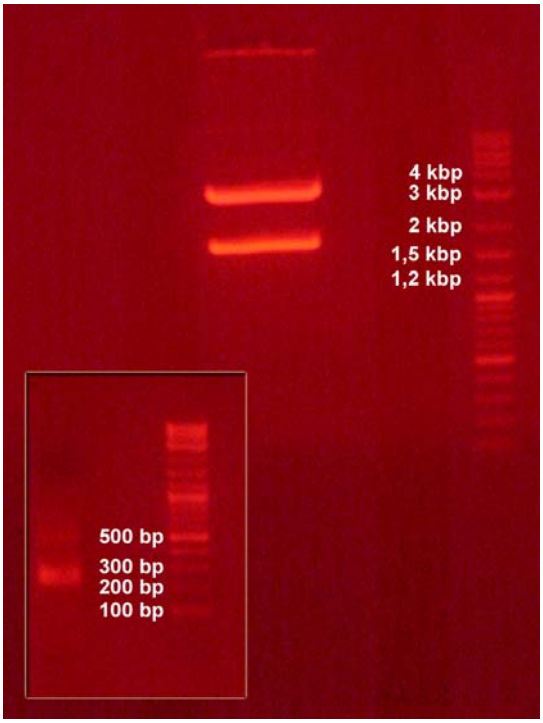


Figure 7.2: *Agarose gel pictures of a sample Sfi I digest reaction. Main picture shows the digest of pComb3H (lane in the middle). Opened vector appears at approx. 3kbp (upper band), the cut out stuffer segment between 1.5 and 2kbp (lower band). Right lane: 2-log DNA marker.*

Inset picture shows the digest of a stem loop DNA fragment (left lane). Product appears between 200 and 300bp (correct size: 230bp). Right lane: 2-log DNA marker.

Sfi I digests of PCR products and the vector pComb3H or pC3MBP were performed to obtain proper sticky ends for ligation. In separate reactions, 2-3µg vector and 1µg *stem loop* DNA, respectively, were incubated with 80U Sfi I for 2.5h at 50°C in a volume of 40µL. Products were purified via agarose gel electrophoresis (Figure 7.2), and the DNA was isolated using centrifuge filter units and ethanol precipitation or a Qiaquick gel extraction kit. After determination of DNA concentrations, DNA was stored at -20°C.

7.3.5.3 Ligation of *Stem Loop* DNA into Vectors and Transfection of *E. coli*

100ng vector and 25ng *stem loop* DNA (both Sfi I digested) were incubated with 200U T4 DNA ligase at RT in a volume of 10µL over night. Ligation reactions without insert DNA served as negative controls, and ligations of vector DNA with a stuffer segment, that was cut out of the vector before, served as positive controls. Electrocompetent *E. coli* were transfected with the ligated vector DNA (ER2537 or ER2738 with pComb3H and XL1-blue with pC3MBP, respectively). Therefore, 1µL of the ligation reaction was mixed with 50µL freshly thawed electrocompetent cells and stored on ice for 1min in a 2mm electro cuvette. After electroporation with 2.5kV, the cuvette was immediately flushed with 900µL prewarmed SOC medium and cell culture was shaken for 20min at 37°C and 200rpm. To titer transformed bacteria, 100 and 10µL of each culture, respectively, were plated on carbenicillin agar plates and allowed to grow over night at 37°C.
A certain number of colonies were analyzed via a PCR colony scan and DNA sequencing to recover clones that possess the *stem loop* DNA fragment of right sequence. The vector DNA of this clone was amplified via plasmid DNA isolation from cell cultures (Section 7.3.3.1) and stored at -20 °C.

7.3.6 Library Construction and Optimization

To evaluate background and diversity of the *stem loop* library, small amounts of library DNA were produced and analyzed. Subsequently, the library size was optimized with large amounts of library DNA. The following randomized oligonucleotides, that possess phosphate at the 5'-end, were purchased from MWG Biotech and applied for library construction (codons are in reading frame):

sense strand: 5'-AG CTT GAG AAG NNK NNK AAA AAG NNK NNK GAA NNK NNK TCC TCC CTC GAA G-3'

anti-sense strand: 5'-C CTC GAG GGA GGA MNN MNN TTC MNN MNN CTT TTT MNN MNN CTT CTC A-3'

7.3.6.1 Evaluation of Background and Diversity

Vector digest: 2µg (o)-phagemid were incubated with 80U Hind III, 80U Sac I, and BSA (100µg/ml) in NE-buffer 1 (supplied with restriction enzyme Sac I) for 3h at 37°C. The reaction was worked up by agarose gelelectrophoresis, and both the phagemid DNA as well as the small DNA fragment (insert) were isolated from the gel, using centrifuge filter units and ethanol precipitation. DNA samples were dissolved in 20µL deionized water each and stored at -20°C.

Annealing of randomized oligonucleotides: 15ng of both of the randomized oligonucleotides were incubated at 95°C for 15min in a volume of 4.5µL. In between, condensation water was centrifuged down several times. Remaining in the heat block, the annealing sample cooled down slowly to RT.

Ligation and transfection of ER2537: The annealing reaction mixture was incubated with 100ng digested (o)-phagmid DNA and 200U T4 DNA ligase over night at RT in a volume of 10µL. Ligation reactions without insert DNA served as negative controls and ligations of vector DNA with the non-randomized DNA insert, that was cut out of the vector before, served as positive controls.
Electrocompetent ER2537 were transfected with the ligated vector DNA. Therefore, 1µL of ligation reaction was mixed with 50µL freshly thawed electrocompetent cells and stored on ice for 1min in a 2mm electro cuvette. After electroporation with 2.5kV, cuvette was immediately flushed with 900µL prewarmed SOC medium and cell culture was shaken for 20min at 37°C and 200rpm. To titer transformed bacteria, 100, 10, and 1µL of each culture, respectively, were plated on carbenicillin agar plates and allowed to grow over night at 37°C. The colony counts on the plates revealed 3.4% background ligation. Single clones of 20 library-colonies were analyzed in detail via PCR colony scan and DNA sequencing in order to evaluate the diversity of the *stem loop* library.

7.3.6.2 Optimization of Library Size

Several parameters of the library production protocol had been varied and optimized. Only the procedure, that was most efficient and resulted in best library size, is described in this

section. Since large amounts of vector DNA is needed for library construction, a plasmid maxipreparation of the (o)-phagemid vector was performed.

Vector digest and purification: 100µg (o)-phagemid were incubated with 400U Hind III, 400U Sac I, and BSA (100µg/mL) in NE-buffer 1 (supplied with restriction enzyme Sac I) for 3h at 37°C in a volume of 400µL. The reaction was worked up by agarose gel electrophoresis and both the phagemid DNA as well as the small DNA fragment (insert) were isolated from the gel and purified by electroelution and subsequent ethanol precipitation. Each DNA sample was dissolved in 20µL deionized water.

Annealing and digest of randomized oligonucleotides: 5 x 300ng of both randomized oligonucleotides were incubated at 95°C for 20min in a volume of 400µL (concentration of both oligonucleotides: approx. 80nM) each sample. In between, condensation water was centrifuged down several times. Remaining in the heat block, the annealing sample cooled down slowly to RT (approx. 1h).
Samples were divided by two and all 10 aliquots á 200µL were incubated with 80U Hind III, 80U Sac I, and BSA (100ng/µL) in NE-buffer 1 for 3h at 37°C in a volume of 300µL. After ethanol precipitation and dissolving in 20µL deionized water, all DNA samples were pooled, and concentration was determined.

Ligation and transfection of ER2537: 75µL (1µg) of the randomized DNA fragment were incubated with 1µg digested (o)-phagemid DNA and 4000U T4 DNA ligase over night at RT in a volume of 200µL. The ligation reaction of vector DNA with the non-randomized DNA insert, that was cut out of the vector before, served as positive control. Ligated phagemid DNA was purified by ethanol precipitation and dissolved in 15µL deionized water.

Electrocompetent ER2537 were transfected with the library DNA. Therefore, all 15µL of ligated (o)-phagemid were mixed with 300µL freshly thawed electrocompetent cells and transferred to a 2mm electro cuvette. After electroporation with 2.5kV, cuvette was immediately flushed with 1mL prewarmed SOC medium, cuvette was rinsed with additional 4mL SOC media and all five fractions were pooled. Cell cultures were shaken for 1h at 37°C and 200rpm. To titer transformed bacteria, 100, 10, and 1µL of each culture, respectively, were plated on carbenicillin agar plates and allowed to grow over night at 37°C. 1µL, 0.1µL, and 0.01µL of each sample were plated on carbenicillin agar plates, and bacteria were allowed to grow over night at 37°C. Colonies were counted and library size was calculated.

7.3.7 Preparation of Helper Phage

Helper phage preparations were either provided by the laboratory of Prof. Barbas at TSRI (1 x 10^{12} - 1 x 10^{13}pfu/ml) or were prepared at the Free University Berlin according to the following protocol:

Phage infection: 2µL of electrocompetent ER2738 were inocculated in 2mL prewarmed SB media and allowed to grow for 1h at 37°C and 200rpm agitation. 1µL of 10^{-6}, 10^{-7}, and 10^{-8} dilutions of commercially obtained VCSM13 stock solution were added to 50µL aliquots of the cell culture and infection proceeded for 15min at RT. All 50µL of each phage-infected cell culture were mixed with 3mL liquified LB top agar (<50°C) and poured on prewarmed plain agar plates. Bacteria were allowed to grow over night at 37°C. Plaques on plates were counted and a phage concentration of 1,4 x 10^{10}pfu/ml was determined for the VCSM13 stock solution.

Phage amplification: For amplification of phage plaques, 2 x 2µL of electrocompetent ER2738 were inocculated in 2mL prewarmed SB media each and allowed to grow for 1h at 37°C and 200rpm agitation. Cell cultures were infected with one phage plaque each and shaken for additional 2h at 37°C and 200rpm agitation. 2mL cultures were transferred to 100mL prewarmed SB media in 250mL Erlenmeyer flasks each and 140µL kanamycin (50mg/mL) were added. Cultures were shaken over night at 37°C / 200rpm.

Phage preparation: 100mL over night cultures were transferred into two 50mL greiner tubes each, and bacteria were centrifuged for 15min at 2500g. Supernatants were transferred to fresh 50mL tubes and incubated at 70°C (water bath) for 20min. Samples were centrifuged at 2500g for 15min, supernatants were transferred to fresh 50mL tubes, and helper phage preparations were stored at 4°C.

Titering of helper phage preparations: 2µL of electrocompetent ER2738 were inocculated in 2mL prewarmed SB media and allowed to grow for 1h at 37°C / 200rpm. 3 x 50µL of the cell culture were infected with 1µL of 10^{-2}, 10^{-5}, or 10^{-8} dilutions of the helper phage preparation for 15min at RT. Phage-infected bacteria samples were mixed with 3mL liquified LB top agar (<50°C) each and poured on plain agar plates. Bacteria were allowed to grow over night at 37°C. Plaques were counted and a phage concentration of 1,22 x 10^{10}pfu/ml was determined for the helper phage preparation. The concentration was very low (should be in the range between 1 x 10^{12} and 1 x 10^{13}pfu/mL), but higher values could neither be achieved in further attempts of helper phage preparation.

7.3.8 Phage and Protein Production

Recombinant phage as well as pIII and MBP fusion proteins were prepared, starting from plasmids with incorporated *stem loop*-encoding DNA.

7.3.8.1 Preparation of pIII and MBP Fusion Proteins

Protein expression: One XL1-blue colony that was transfected with the appropriate pC3MBP clone, or one ER2738 colony that was transfected with the appropriate pComb3H clone, respectively, was picked from a carbenicillin agar plate (from titering after electroporation) and inocculated in 3mL prewarmed SB media. Bacteria were allowed to grow for 8h at 37°C / 200rpm agitation. Overexpression of the MBP fusion protein was induced by addition of IPTG to a final concentration of 1mM and protein production proceeded over night at 37°C / 200rpm.

Protein preparation: Cell cultures were centrifuged for 10min at 3000g / 4°C, and supernatants were discarded. Cells were resuspended in 250µL PBS and lyses were performed by alternating incubation at -80°C and 37°C for 3min each (6x). Cell fragments were precipitated by centrifuging for 5min at 15000g / 4°C. The supernatants of the cell lysates were stored at -80°C and used for binding assays (MBP fusion proteins) or PAGE analyses (pIII fusion proteins) without any further isolation or purification.

7.3.8.2 Production of Recombinant Phage

Production of recombinant phage either started from transfection of *E. coli* (ER2537 or ER2738) with phagemid vector by electroporation or reinfection of *E. coli* (ER2537 or ER2738) with recombinant phage. For some binding tests, the target binding proceeded during small-scale phage production right after release of the particle from cell.

Phage Production Starting from Transfection:

Phage used in binding tests and phage libraries prepared for panning procedures, respectively, were produced by transfection of phagemid DNA. Therefore, purified pComb3H clones or library DNA were introduced into bacteria, applying the electroporation protocols that have been described in sections 7.3.6.1 (single clone DNA) and 7.3.6.2 (library DNA),

respectively. After electroporation and dilution with SOC media, transfected bacteria were shaken for 1h at 37°C / 200rpm agitation.

Production of single clone phage: 2mL prewarmed SB media and 0.6µL carbenicillin (100mg/mL) were added to 900µL cell culture. After 1h incubation at 37°C / 200rpm agitation, additional 0.8µL carbenicillin (100mg/mL) were added and incubation proceeded for 1h at 37°C / 200rpm. Cell cultures were transferred to 97mL prewarmed SB media, containing 46µL carbenicillin (100mg/mL) in centrifuge bottles, and 1mL helper phage was added. After incubation for 3h at 37°C / 200rpm, 140µL kanamycin (50mg/mL) were added and phage were produced over night at 37°C / 200rpm agitation.

Production of library phage: 10mL prewarmed SB media and 3µL carbenicillin (100mg/mL) were added to 5mL cell culture, and the library was titered by plating 1µL and 0.1µL on carbenicillin agar plates. After 1h incubation at 37°C / 200rpm agitation, additional 4.5µL carbenicillin (100mg/mL) were added, and the incubation proceeded for 1h at 37°C / 200rpm. Cell cultures were transferred to 183mL prewarmed SB media, containing 92.5µL carbenicillin (100mg/mL) in centrifuge bottles, and 2mL helper phage was added. After incubation for 2h at 37°C / 200rpm, 280µL kanamycin (50mg/mL) were added and phage were produced over night at 37°C / 200rpm agitation.

Phage Production Starting from Reinfection:

Phage production via reinfection of recombinant phage was either performed with phage preparations to reamplify phage libraries and produce single clone phage for binding tests, respectively, or proceeded during panning procedures to amplify selected phage clones.

Reinfection of phage preparations: (For library reamplification, double volumes were used): 7.5µL electrocompetent *E. coli* were inocculated in 5mL prewarmed SB media and allowed to grow for 2h at 37°C and 200rpm agitation. Bacteria were infected with 5µL phage preparation and incubation proceeded for 1h at 37°C / 200rpm. After addition of 5mL prewarmed SB media and 2.5µL carbenicillin (100mg/mL), samples were shaken for 1h at 37°C / 200rpm. Cell cultures were transferred to 90mL prewarmed SB media, containing 46µL carbenicillin (100mg/mL) in centrifuge bottles and 1mL helper phage was added. After incubation for 2h at 37°C / 200rpm, 140µL kanamycin (50mg/mL) were added and phage were produced over night at 37°C / 200rpm agitation.

Reinfection of selected phage clones: 7.5µL electrocompetent *E. coli* were inocculated in 5mL prewarmed SB media and allowed to grow for 2h at 37°C and 200rpm agitation. Bacteria were infected with 100µL phage elution sample and the incubation proceeded for 30min at 37°C / 200rpm. 5mL prewarmed SB media and 2µL carbenicillin (100mg/mL) were added, and the cell culture was shaken for additional 30min at 37°C / 200rpm (in between, the output titering was performed by plating 1µL and 0.1µL of reinfected bacteria on carbenicillin agar plates). 1mL helper phage solution was added, and the cell culture was transferred to 90mL prewarmed SB medium, containing 46µL carbenicillin (100mg/mL) in centrifuge bottles. After 1.5h incubation at 37°C / 200rpm, 140µL kanamycin (50mg/mL) were added and the phage were produced over night at 37°C / 200rpm.

Phage Production for Real-Time Binding Assays:

For real-time binding assays, the binding of target peptides proceeded in the phage production culture. After transfection of phagmid DNA into ER2738, applying the standard protocol for library phage production, 5mL cell culture was incubated at 37°C / 200rpm for 1h. 10mL prewarmed SB media and 3µL carbenicillin (100mg/mL) were added and bacteria were shaken for 30min at 37°C / 200rpm. After addition of 4.5µL carbinicillin (100mg/mL) and incubation at 37°C / 200rpm for additional 30min, 1mL cell culture was transferred to a 2mL centrifuge tube, and the helper phage solution was added. Three different protocols were applied for phage preparation and target-binding:

I) After infection with 20µL helper phage solution, bacteria were shaken for 30min at 37°C / 1000rpm, and the target peptide was added to different final concentrations (10µM, 1µM, 100nM, 10nM). The binding proceeded for 1h at 37°C / 1000rpm, and cells were centrifuged for 30min at 7000rpm / 4°C using a table-top centrifuge. The supernatant was transferred to 115µL precooled 20% PEG / 2.5M sodium chloride solution, and the phage precipitated on ice for 30min. The samples were centrifuged for 30min at 13000rpm / 4°C, using a table-top centrifuge, and the phage pellets were dried, resuspended in 100µL 1% BSA in PBS, and used in *reversed phage ELISA* tests.

II) After infection with 100µL helper phage solution, bacteria were shaken for 30min at 37°C / 1000rpm, and the target peptide was added to different final concentrations (10µM, 1µM, 100nM, 10nM). The binding proceeded for 1h at 37°C / 1000rpm, and cells were centrifuged for 30min at 7000rpm / 4°C, using a table-top centrifuge. 100µL of supernatant were used in *reversed phage ELISA* tests without precipitation of the phage.

III) After infection with 50µL helper phage solution and addition of the target peptide to different final concentrations (10µM, 1µM, 100nM, 10nM), the binding reaction proceeded at 37°C / 1000rpm. After definite times (45min, 90min, and 145min), fresh target peptide was added. After 3h, cells were centrifuged for 30min at 6000rpm / 4°C, using a table-top centrifuge, and 100µL of supernatant were used in *reversed phage ELISA* tests without precipitation of the phage.

7.3.8.3 Preparation of Recombinant Phage

Over night cultures were centrifuged at 3000g / 4°C for 30min. The supernatant was transferred to precooled centrifuge bottles, containing 0.2 volumes 20% PEG 8000 / 2.5M sodium chloride solution, and the phage precipitation proceeded for 30min on ice. The phage were centrifuged for 30min at 15000g / 4°C. The supernatant was discarded, and bottles were drained by inverting on a paper towel for 10min. Remaining liquids were wiped off from the upper part of the centrifuge bottles with paper towels. Phage pellets were resuspended with 0.02 volumes (according to over night phage culture) buffered solution (pH 7.4), and passed through a 0.22µm filter. For storage at 4°C, sodium azide was added to a final concentration of 0.02% (w/v).
The composition of the phage resuspension solution depended on the application of the phage preparation. Normaly TBS or PBS were used. These buffers could contain 5mM – 50mM DTT and 1% BSA (w/v), respectively. For some binding tests, the phage were resuspended in 6M guanidine hydrochloride.

7.3.9 Evaluation of the Display of Recombinant pIII on Phage Coat

Several experiments, such as SDS-PAGE with Western Blotting, *ELISA* tests, and binding assays on magnetic particles were performed in order to investigate the presentation of the pIII-*stem loop* fusion protein on the surface of the respective phage clones.

7.3.9.1 Detection of *Stem Loop*-Cys Residues on (t)- and (nt)-phage

For the detection of free SH-groups on the N-terminal cysteine residue of the *stem loop* protein fragment on phage surface, Maleimide-*ELISA* tests, applying two different strategies, were performed.

Reversed Phage-Maleimide ELISA (Figure 5.31a):

Main protocol: *ELISA* plate wells were coated with 25µL of a maleimide-BSA solution (4µg/mL) for 2h at 37°C. Plates were washed 3 x with deionized water. Free protein binding sites were blocked with 175µL 3% BSA in PBS per well for 1h at RT without subsequent washing. The maleimide-phage reaction proceeded with 25µL phage preparation per well for 1h at 37°C. After binding, plates were washed 3 x with deionized water. For phage detection, the binding of 25µL 1:5000 dilution of anti-M13 HRP-conjugated antibody per well proceeded for 1h at 37°C. Plates were washed 8 x with deionized water. The enzyme-color reaction was performed with 25µL HRP developer per well for 1h at RT and OD_{405} was measured using an *ELISA* plate reader.

Specific assay conditions: (o)-Phage were used as negative controls. The phage preparation was made in 6M guanidine hydrochloride.

Variable assay parameters (combinatorial system): Maleimide-phage reactions were performed with different concentrations of guanidine hydrochloride. Therefore, the phage preparation was diluted 1:12 in respective PBS / GdnHCl mixtures. Maleimide-phage reactions were performed with or without DTT. The following steps of the assay were skipped in negative controls: maleimide-BSA coating and BSA blocking, respectively.

Phage-Maleimide ELISA (Figure 5.31b):

Main protocol: *ELISA* plate wells were coated with 25µL of an anti-M13 antibody solution (5ng/well) for 1h at 37°C. Plates were washed 3 x with deionized water. Free protein binding sites were blocked with 175µL 3% BSA in PBS per well for 1h at RT without subsequent washing. Phage binding was performed with 25µL phage solution per well for 2h at RT, followed by 10 x washing with deionized water. The maleimide-phage reaction proceeded with 25µL maleimide-HRP solution for 1h at RT. Plates were washed 10 x with deionized water. The enzyme-color reaction was performed with 25µL HRP developer per well for 1h at RT and OD_{405} was measured using an *ELISA* plate reader.

Specific assay conditions: (o)- and (no)-Phage, respectively, were used as negative controls. The phage preparation was made in 6M guanidine hydrochloride. 5mg Maleimide-HRP were dissolved in 1mL 100mM phosphate buffer pH 7.2 and mixed with 1mL glycerol. The stock solution was stored at -20°C.

Variable assay parameters (combinatorial system): Maleimide-phage reactions were performed with different concentrations of guanidine hydrochloride. Therefore, the phage preparation was diluted 1:6 in respective PBS / GdnHCl mixtures. Maleimide-phage reactions were performed without or with varying disulfide reducing reagents and with different dilutions of the maleimide-HRP stock solution (1:10, 1:100, 1:1000, 1:10000). The following steps of the assay were skipped in negative controls: antibody coating and BSA blocking, respectively.

7.3.9.2 SDS-PAGE and Western Blotting of (nt)-Phage

PIII fusion proteins of (nt) and (no), respectively, had either been prepared by overexpression from ER2738 or by denaturation of phage preparations. Proteins were separated by polyacrylamide gel electrophoresis and detected by western blotting (Figure 5.36). To assure equal treatment of all sample proteins, the phage denaturation procedure was performed also with pIII fusion proteins that were prepared by overexpression from E. coli.

Titers of the phage samples used: 7.7×10^{12} (nt) and 13.7×10^{12} (no)

Phage precipitation and denaturation: Prior to denaturation, 1mL of phage preparation was incubated with 125µL 20% PEG 8000 / 2.5M sodium chloride solution, and the phage precipitation proceeded for 30min on ice. Phage were centrifuged at 14000rpm, using a table-top centrifuge, the supernatant was discarded, and phage were resuspended in 10µL deionized water. 10µL of each protein sample were mixed with 10µL SDS loading buffer and incubated for 10min at 95°C.

SDS-Polyacrylamide gel electrophoresis: 20µL of each protein sample were loaded on a 12% acrylamide gel and the electrophoresis was performed for 2h at 150V in SDS PAGE buffer. The gel was prepared from the following components:

Component	Resolving Gel	Stacking Gel
deionized water	1.6mL	1.4mL
30% acrylamide mix	2.0mL	0.33mL
Tris buffer	1.3mL 1.5M pH 8.8	0.25mL 1.0M pH 6.8
10% SDS	50µL	20µL
10% APS	50µL	20µL
TEMED	2µL	2µL

Western Blot: The gel was prepared for blotting by soaking in electroblotting buffer for 20min. The PVDF Hybond-P membrane was prepared by pre-wetting in methanol for 10sec, subsequent washing in deionized water for 5min, and equilibrating in electroblotting buffer for additional 10min. The blotting procedure was performed in electroblotting buffer for 1.5h at 125mA / 30V with the following packing in blot-aparature (from kathode to anode): 3 slices of filter / gel / membrane / 3 slices of filter. After blotting, the gel was stained with Coomassie staining solution for 1h at RT on an orbital shaker, subsquently destained for 2 x 45min in 30% methanol / 10% acetic acid and stored at 4°C in saran wrap.

Protein detection: The blocking of non-specific binding sites was performed by immersing the membrane in 5% non-fat dried milk in TBS-T for 1h at RT on an orbital shaker. The membrane was rinsed briefly with 2 changes of TBS-T. For binding of the primary antibody, the mouse-anti-M13 pIII-antibody, that binds the C-terminal region (aa 259-406) of wild type and recombinant pIII, was diluted 1:10000 in PBS-T, and the membrane was incubated for 1h at RT on an orbital shaker. After binding, the membrane was rinsed, 15min incubated and subsequently 3 x 5min washed with fresh changes of TBS-T. For binding of the secondary antibody, the goat-anti-mouse HRP-conjugated antibody was diluted 1:10000 in PBS-T, and the membrane was incubated for 1 h at RT on an orbital shaker. After binding, the membrane was rinsed, 15min incubated, and subsequently 3 x 5min washed with fresh changes of TBS-T.
Visualization of pIII proteins was performed on an autoradiographic film with an ECL Plus Western Blotting detection kit, applying the experimental protocol provided by the supplier. Different exposure times were tried.

7.3.9.3 Detection of MBP-*Stem Loop* Fusion Proteins on (ft)-Phage

Main protocol (*phage-maleimide ELISA*, Figure 5.39): *ELISA* plate wells were coated with 25µL of an anti-M13 antibody solution (5ng/well) for 2h at 37°C. Plates were washed 2 x with deionized water. Free protein binding sites were blocked with 175µL 3% BSA in PBS per well for 1h at RT without subsequent washing. The phage binding was performed with 25µL of phage dilution per well for 1h at 37°C, followed by 2 x washing with deionized water. The binding of the primary antibody was performed with 25µL of an mouse-anti-MBP antibody solution (25ng/well) for 30min at 37°C and 10 x subsequent washing with deionized water. The binding of the secondary antibody was performed with 25µL of an anti-mouse AP-conjugated antibody (25ng/well) for 30 min at 37°C. Plates were washed 10 x with deionized water. The enzyme-color reaction was performed with 25µL AP developer / PNPP per well for 1h at 37°C, and OD_{405} was measured using an *ELISA* plate reader.

Specific assay conditions: (no)-Phage were used as negative controls. Phage preparations were made in PBS. 1 PNPP tablet was dissolved in 5mL AP developing buffer prior to use (recipe for one plate). A dilution series of phage (2^n) was used for phage capture reaction.

Variable assay parameters (combinatorial system): The phage capture step was performed with 5mM DTT or without any disulfide reducing reagent.

7.3.9.4 Detection of the FLAG tag on (et)-Phage

Binding assay: Phage cultures were incubated with 10mM DTT for 1h at 37°C / 200rpm prior to spinning of bacteria, and a standard phage preparation was performed (Section 7.3.8.3). Pellets were resuspended in 1.5mL TBS. The FLAG-detection assay was performed on streptavidin-coated magnetic particles. For binding of an biotinylated anti-FLAG antibody, 30µL of magnetic particles were incubated with 500µL TBS, containing 1.2µg antibody for 1h at RT. Particles were washed twice with 500µL TBS-T (4min incubation). The blocking proceeded with 500µL 5% non-fat dried milk in TBS for 1h at RT, and particles were washed twice with 500µL TBS-T (4min incubation). The phage binding on magnetic particles was performed with 500µL phage preparation for 1.5h at RT. Particles were washed 9 x with 500µL TBS-T and once with 500µL TBS (4min incubation).

Reinfection: *E. coli* for phage infection were prepared by inocculation of 7.5µL electrocompetent ER2738 in 5mL prewarmed SB media and growing for 1.5-2h at 37°C / 200rpm. Elution of bound phage from magnetic particles proceeded with 25µL freshly prepared trypsin solution (10mg/mL in TBS) for 30min at RT, and the reaction was quenched with 75µL SB media. For reinfection, 100µL phage solution was transferred to 5mL *E. coli* culture and shaken for 30min at 37°C / 200rpm agitation.

Determination of phage titers: The input-titering was performed by infecting 50µL *E. coli* with 1µL of an 1 x 10^{-7} dilution of the phage preparation and incubation for 15min at RT. All 50µL were plated on carbenicillin agar plates, and bacteria were allowed to grow over night at 37°C. Output-titering was performed by diluting 5µL of the 5mL reinfection cell culture in 195µL SB media. 100µL were plated on carbenicillin agar plates, and bacteria were allowed to grow over night at 37°C. After determination of colony counts, output / input ratios for all samples were calculated.

7.3.10 *Stem Loop* - Target Binding Assays

To evaluate the binding of the electrophilic peptide fragment to phage-diplayed *stem loop* protein fragments, binding assays were performed, that followed different strategies. Either *ELISA* tests, using 96-well plates and enzyme-color reactions for quantification of *coiled coil* formation were performed, or binding was investigated on magnetic particles with subsequent elution of bound phage, reinfection of E. coli, and quantification via phage titering.

7.3.10.1 Evaluation of *Coiled Coil* formation using *ELISA* Binding Tests

Two main *ELISA* strategies were utilized to evaluate the binding of the electrophilic peptide fragment to (t)-, (nt)-, (nt-MBP)-, and (ft)-phage. In a *reversed phage ELISA*, phage were detected directly via HRP-conjugated antibodies, while well plates were coated with phage via antibodies in a *phage ELISA* tests.

Reversed Phage ELISA (Figures 5.28 and 5.29):

Main protocol: Plate wells were coated with 25µL strepatvidine (8µg/mL in PBS) per well for 1h at 37°C, and plates were washed twice with deionized water. The blocking of non-bound protein binding sites proceeded with 175µL 3% BSA in PBS for 1h at 37°C without subsequent washings. The exeprimental protocol for the following steps depended on the *coiled coil* binding strategy that was applied.

Solid phase binding (Figure 5.28): 25µL Biotinylated target peptides (5µM) per well were bound to streptavidin for 30min at RT, and plates were washed twice with deionized water. The *coiled coil* formation started with addition of 25µL phage solution per well and proceeded for variable time spans. Subsequently, plates were washed 10 x with solutions of varying composition.

Solution phase binding (Figure 5.29): The phage-target binding was performed in solution in 1.5mL centrifuge tubes with varying parameters and reaction conditions. 25µL binding reaction were transferred into each plate well, and bound phage were captured for 1h at RT. Subsequently, plates were washed 10 x with solutions of varying composition.

For phage detection, 25µL anti-M13 HRP-conjugated antibody (5ng/well) were added and binding was performed for 1h at 37°C. Plates were washed 8 x with deionized water. The enzyme-color reaction proceeded with 25µL HRP developer / well for 1h at RT, and the quantification of *coiled coil* binding was performed via OD_{405} measurement using an *ELISA* plate reader.

Variable assay parameters and conditions: Several assay parameters and binding conditions have been varied systematically and in a combinatorial manner in the *ELISA* tests that were performed:

Phage-target binding: Binding reactions were performed for varying time spans (1h – 24h) and at different temperatures (RT / 30°C / 37°C). Either no disulfide reducing reagents or DTT or 2-mercaptoethylamine were used. As detergents, either Tween 20 (0.1%-2%) or guanidine hydrochloride (0.5-3M) were added. Therefore, some phage samples were prepared in 6M GdnHCl and incubated for 1h / 24h prior to binding reaction. A protease inhibitor (complete) was used to prevent proteolytic degradation of phage-displayed *stem loop*. During solution phase binding procedures, the *coiled coil* formation proceeded with varying concentrations of target peptide (10nM – 100µM). Binding reactions were either used directly for immobilization in different dilutions (1:1 – 1:1000 in PBS), or phage were precipitated and resuspended in PBS before. Also phage from *real-time binding reactions* were tested in *reversed phage ELISAs*.

Washing after phage capture: Washing steps were either performed with deionized water or with PBS, containing detergents like Tween 20 (0.1%-2%) and / or GdnHCl (0.5-2M) to remove unspecifically bound phage. 10mM DTT was used to reduce disulfide bonds and hydroxylamine to cleave thioester bonds between electrophilic peptide fragments and SH-groups on phage surface.

Phage ELISA (Figure 5.30):

Main protocol: *ELISA* plate wells were coated with 25µL of an anti-M13 antibody solution (5ng/well) for 2h at 37°C. Plates were washed 3 x with deionized water. Free protein binding sites were blocked with 175µL 3% BSA in TBS per well for 1h at 37°C without subsequent washing. The phage binding was performed with 25µL of the phage dilution per well for 1h at 37°C, followed by 3 x washing with deionized water. *Coiled coil* binding proceeded with 25µL biotinylated target peptide solution (5µM in PBS) per well for 1h at 37°C and plates were washed 10 x with solutions of varying composition. The labeling of the bound target peptide

was performed with 25µL HRP-conjugated streptavidin solution (4µg/mL) per well for 1h at 37°C. Plates were washed 5 x with deionized water. The enzyme-color reaction proceeded with 25µL HRP developer / well for 1h at RT, and the quantification of *coiled coil* binding was performed via OD_{405} measurement using an *ELISA* plate reader.

Variable assay parameters and conditions: Several assay parameters and binding conditions have been varied systematically in a combinatorial manner in the *ELISA* tests that were performed:

The phage capture proceeded with varying concentrations of guanidine hydrochloride (0.25-6M) and either without disulfide-reducing reagent or with 5mM DTT. The washing after target binding was performed with PBS or with 0.5% Tween in PBS. In an alternative procedure, phage were coated directly to plate wells without anti-M13 antibody.

Reversed MBP ELISA (Figure 5.34):

Main protocol: Plate wells were coated with 25µL strepatvidine (8µg/mL in PBS) per well for 1h at 37°C and plates were washed twice with deionized water. 25µL biotinylated target peptides (5µM) per well were bound to streptavidin for 1h at 37°C, and plates were washed twice with deionized water. The blocking of non-bound protein binding sites proceeded with 175µL 3% BSA in PBS for 1h at 37°C without subsequent washings. The *stem loop*-target binding was performed with 25µL dilution of a cell extract per well (2^n dilution series in PBS) for 2h at RT. Plates were washed 10 x with deionized water. The binding of the primary antibody was performed with 25µL of an mouse-anti-MBP-antibody solution (25ng/well) for 30min at 37°C and 10 x subsequent washing with deionized water. The binding of the secondary antibody was performed with 25µL of an anti-mouse AP-conjugated antibody (25ng/well) for 30 min at RT. Plates were washed 10 x with deionized water. The enzyme-color reaction was performed with 25µL AP developer / PNPP per well for 30min at 37°C, and OD_{405} was measured using an *ELISA* plate reader.

Specific assay conditions: 1 PNPP tablet was dissolved in 5mL AP developing buffer prior to use (recipe for one plate). A dilution series of phage (2^n) was used for the phage capture reaction.

7.3.10.2 Evaluation of *Coiled Coil* Formation using Magnetic Particles

Binding tests using streptavidin-coated magnetic particles were performed to evaluate *coiled coil* formation of the electrophilic target peptide with (at)- and (et)-phage. After elution of captured phage from particles, bacteria were reinfected and phage were titered.

Binding assay: For the immobilization of target peptides, 30µL of streptavidin-coated magnetic particles were incubated with 500µL 10µM biotinylated peptide in PBS for 1h at RT. Particles were washed once with 500µL 0.5% Tween 20 in PBS and once with PBS (4min incubation). The blocking proceeded with 500µL 5% non-fat dried milk in PBS for 1h at RT. The target-phage binding on magnetic particles was performed with 500µL phage solution for 1-2h at RT. Particles were washed 9 x with 500µL 0.5% Tween 20 in PBS and once with 500µL TBS (4min incubation).

Reinfection: *E. coli* for phage infection were prepared by inocculation of 7.5µL electrocompetent ER2738 in 5mL prewarmed SB media and growing for 1.5-2 h at 37°C / 200rpm. The elution of bound phage from magnetic particles proceeded with 25µL freshly prepared trypsin solution (10mg/mL in TBS) for 30min at RT and the reaction was quenched with 75µL SB media. For reinfection, 100µL phage solution was transferred to 5mL *E. coli* culture and shaken for 30min at 37°C / 200rpm agitation.

Determination of phage titers: Input-titering was performed by infecting 50µL *E. coli* with 1µL of an 1 x 10^{-7}-1 x 10^{-8} dilution of the phage preparation and incubation for 15min at RT. All 50µL were plated on carbenicillin agar plates and bacteria were allowed to grow over night at 37°C. Output-titering was performed by diluting 2µL of the 5mL reinfection cell culture in 198µL SB media. 100µL were plated on carbenicillin agar plates and bacteria were allowed to grow over night at 37°C. After determination of colony counts, output / input ratios for all samples were calculated.

Specific conditions for (at)-phage: In binding tests with (at)-phage and target peptides with or without C-terminal thioester, washings after phage capture were performed with 0.01% Tween in PBS.

Specific conditions for (et)-phage: For the evaluation of (et)-binding, phage were incubated with enterokinase light chain (EK_L) to remove the N-terminal tag of the *stem loop* protein fragment. Several assay parameters have been varied systematically in a combinatorial manner to accomplish tag removal by EK_L:

Phage cultures were incubated with different concentrations of DTT (0-25mM) for 1h at 37°C / 200rpm prior to centrifuging of bacteria or during phage precipitation on ice, and a standard phage preparation was performed (Section 7.3.8.3). Pellets were resuspended in 1,5mL PBS, containing different concentrations of DTT (0-50mM). The tag-cleavage reaction was performed at varying temperatures (RT / 37°C) with varying amounts of EK_L (0.0125 – 125 U) and different time spans (1h – 24h).

7.3.10.3 Phage Binding during Selection Rounds

Selection procedures with phage libraries comprised standard protocols for phage production (Section 7.3.8.2) and preparation (Section 7.3.8.3) as well as a target-phage binding procedure. These selections were performed in solution, and the capture occured on streptavidin-coated magnetic particles.

Selection procedure: The *coiled coil* binding reaction was performed for 1h at RT in 500µL PBS, containing 1% non-fat dried milk, 100nM biotinylated target peptide and 100µL phage library preparation from transformation or last panning round. Blocking of magnetic particles proceeded with 500µL 5% non-fat dried milk in PBS for 2h at RT. The blocking solution was removed, and the target-phage binding reaction was incubated with the magnetic particles for 1h at 4°C. Particles were washed 10 x with 500µL 2% Tween 20 in PBS and once with 500µL PBS (4min incubation).

Reinfection: *E. coli* for phage infection were prepared by inocculation of 7.5µL electrocompetent ER2537 in 5mL prewarmed SB media and growing for 1.5-2.5 h at 37°C / 255rpm. The elution of bound phage from magnetic particles proceeded with 25µL freshly prepared trypsin solution (10mg/mL in TBS) for 30min at RT, and the reaction was quenched with 75µL SB media. For reinfection, 100µL phage solution was transferred to 5 mL *E. coli* culture and shaken for 30min at 37°C / 255rpm agitation.

Determination of phage titers and phage production: The input-titering was performed by infecting 50µL *E. coli* with 1µL of an 1 x 10^{-9} dilution of the phage preparation and incubation for 15min at RT. All 50µL were plated on carbenicillin agar plates and bacteria were allowed to grow over night at 37°C. The output-titering and the phage production were performed as described in section 7.3.8.2 After determination of colony counts, output / input ratios for all samples were calculated.