Published in final edited form as:

Methods Mol Biol. 2021 ; 2355: 219–230. doi:10.1007/978-1-0716-1617-8_17.

The Construction of a Genetically Encoded, Phage-Displayed Cyclic-Peptide Library

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Abstract

Due to the great potentials of cyclic peptides as therapeutic agents. Several phage-displayed peptide libraries in which cyclization is achieved by the covalent linkage of cysteines have been previously demonstrated to identify cyclic-peptide ligands for therapeutic targets. While problems remain in these cysteine conjugation strategies, we have invented a phage display technique in which its displayed peptides are cyclized through a proximity-driven Michael addition reaction between a cysteine and an amber-codon-encoded *N*^ε-acryloyl-lysine (AcrK). Using a randomized 6-mer library in which peptides were cyclized at two ends through a cysteine–AcrK linker, we demonstrated the successful selection of a potent ligand, Cych8a, for histone deacetylase 8 (HDAC8). We believe this approach will find broad applications in drug discovery.

1. Introduction

Cyclic peptides are considered as great therapeutic agents for their superior performance on biological activities. Many strategies have been developed for the direct selection of cyclic-peptide libraries using phage display [1-10]. However, while challenges remain in these approaches, we proposed an alternative that features a proximity-driven cyclization between a phage-displayed electrophilic noncanonical amino acid (ncAA) AcrK and a cysteine (Fig. 1a). The incorporation of AcrK into phages can be achieved by suppressing an amber mutation in the phage-displayed peptide coding region in *E. coli* that harbors a ncAA-specific aminoacyl-tRNA synthetase–amber suppressor tRNA pair and grows in the presence of the AcrK (Fig. 1b). Using this method for the construction of a phage display library will afford a genetically encoded, phage-displayed cyclic-peptide library whose spontaneous peptide cyclization requires neither the modification of phage strains nor an organic linker for cyclization.

2. Materials

All solutions should be prepared using Milli-Q water (mQ water).

2.1 Growth media, Solutions, Buffers, Antibiotics, Plates

1. 2YT medium: Dissolve 16 g of tryptone, 10 g of yeast extract, and 5 g of NaCl in 1 L of mQ water. Autoclave at 121 °C for 15 min.

2. No salt Luria-Bertani (LB) medium: Dissolve 10 g of tryptone and 5 g of yeast extract in 1 L of mQ water. Autoclave at 121 °C for 15 min.
3. Ampicillin stock solution (100 mg/ml, 1000x): Dissolve 1 g of sodium ampicillin in 10 mL of mQ water. Pass the solution through a 0.22 µm filter to sterilize.
4. Chloramphenicol stock solution (34 mg/ml, 1000x): Dissolve 0.34 g of chloramphenicol in 10 ml of 100% ethanol. Pass the solution through a 0.22 µm filter to sterilize.
5. Kanamycin stock solution (25 mg/ml, 1000x): Dissolve 0.25 g of kanamycin monosulfate in 10 ml of mQ water. Pass the solution through a 0.22 µm filter to sterilize.
6. Tetracycline stock solution (15 mg/ml, 1000x): Dissolve 0.15 g of tetracycline hydrochloride in 10 ml of 70% ethanol/mQ water solution. Pass the solution through a 0.22 µm filter to sterilize.
7. Phosphate-buffered saline (PBS): Dissolve 8 g of NaCl, 200 mg of KCl, 1.44 g of Na₂HPO₄, and 245 mg of KH₂PO₄ in 800 mL of mQ water, adjust pH to 7.4 and add mQ water to bring to a final volume of 1 L.
8. 5x Blocking buffer (5% bovine serum albumin (BSA), 0.1% Tween20 in PBS buffer): Dissolve 0.5 g of BSA and 50 µL of Tween20 in 10 mL of PBS buffer.
9. 1x Blocking buffer (1% BSA, 0.1% Tween20 in PBS buffer): Prepare the solution by diluting 5x blocking buffer with PBS buffer.
10. 1x Wash buffer (0.1% Tween 20 in PBS buffer): Dissolve 50 µL of Tween 20 in 50 mL of PBS buffer.
11. Arabinose stock solution (20%, 100x): Dissolve 20 g of L-arabinose in 80 mL of mQ water. Pass the solution through a 0.22 µm filter to sterilize.
12. Isopropyl β-D-1-thiogalactopyranoside (IPTG) stock solution (1 M, 1000x): Dissolve 2.38 g of IPTG in 10 mL of mQ water. Pass the solution through a 0.22 µm filter to sterilize.
13. 5x phage precipitation solution (2.5 M NaCl, 20% polyethylene glycol (PEG) 8000): Dissolve 73.05 g of NaCl and 100 g of PEG 8000 in 400 mL of mQ water. Put a magnetic stir bar to the solution and add mQ water to bring to a final volume of 500 mL. Autoclave at 121 °C for 15 min, and before the liquid cools down stir the mixture to afford a homogenous solution.
14. Glycine elution solution (50 mM glycine-HCl, pH: 2.2): Dissolve 375 mg of glycine in 80 mL of mQ water, adjust pH with HCl to 2.2. Add mQ to bring it to a final volume of 100 mL.
15. Tris(hydroxymethyl)aminomethane (Tris) neutralization solution (1 M Tris, pH: 8.0): Dissolve 12.11 g of Tris in 80 mL of mQ water, adjust pH with HCl to 8.0. Add mQ to bring it to a final volume of 100 mL.

16. LB agar plates: Mix 10 g of agar, 5 g of NaCl, 5 g of tryptone, 2.5 g of yeast extract and add mQ water to a final volume of 0.5 L. Autoclave at 121 °C for 15 min and allow the agar solution to cool to 50-60 °C. Add the appropriate amount of desired antibiotic(s) to the solution, and transfer 15 mL of LB agar solution per 10 cm Petri dish.

2.2 Cloning, Expression and Selection of the Phage Library

1. Phage display vector pADL10b purchased from Antibody Design Labs.
2. Primers pADL-F and pADL-R that may be purchased as custom DNA oligos from Integrated DNA Technologies.
3. Phusion® High-Fidelity Polymerase Chain Reaction (PCR) Kit purchased from New England Biolabs.
4. Enzymes (e.g., DpnI, NcoI-HF, T4 DNA ligase) that may be purchased from New England Biolabs.
5. DNA kits (e.g., Miniprep, Midiprep, QIAquick PCR purification kit, and QIAquick gel extraction kit) purchased from Qiagen.
6. *E. coli* strains (e.g., Top10, Top10F') for cloning that may be purchased from ThermoFisher.
7. Electroporation cuvettes (2mm).
8. Sera-Mag Streptavidin coated Magnetic Beads purchased from GEHealthcare.
9. MagJET Separation Rack (2 x 1.5 mL tube) purchased from ThermoFisher.

2.3 Synthesis of the Selected Peptide Cych8a

1. *N,N*-Dimethylformamide (DMF)
2. Dichloromethane (DCM)
3. Piperidine/DMF (20% v/v)
4. Methanol
5. Diisopropylethylamine (DIEA)
6. Deprotection solution (Mtt): Trifluoroacetic acid (TFA)/triisopropylsilane (TIS)/DCM (1/5/94, v/v/v)
7. Cleavage cocktail: TFA/TIS/H₂O/2,2'-(ethylenedioxy)diethanethiol (DODT) (92.5/2.5/2.5/2.5, v/v/v/v)
8. 1x PBS buffer

9. Rink amid resin (Loading capacity: 0.54 mmol/g, 100-200 mesh) purchased from Novabiochem.
10. 9-Fluorenylmethyloxycarbonyl (Fmoc) protected amino acids (Fmoc-AA-OH) purchased from CEM.
11. Tetramethyluronium hexafluorophosphate (HBTU)
12. 5-Carboxyfluorescein, single isomer (5-FAM)
13. *N*-Succinimidyl acrylate [11]
14. Kaiser-ninhydrin solutions: Reagent A: Dissolve 16.5 mg of KCN in 25 mL of mQ water. Dilute 1 mL of afforded solution with 49 mL of pyridine. Reagent B: Dissolve 1 g of ninhydrin in 20 mL of n-butanol. Reagent C: Dissolve 40 g of phenol in 20 mL of n-butanol.
15. Solid-phase peptide synthesis (SPPS) vessel (50 mL)

2.4 Characterization of the Selected Peptide Cych8a

1. 96-Well black flat bottom polystyrene plate purchased from Corning
2. Boc-Lys(Ac)-AMC [11]
3. Trichostatin A
4. Trypsin from bovine pancreas

2.5 Instruments

1. Thermocycler
2. MicroPulser Electroporator purchased from Bio-rad.
3. Spectrophotometer
4. Incubator shaker
5. Centrifuge
6. DNA Gel imager
7. High-performance liquid chromatography (HPLC) system
8. Lyophilizer
9. Nuclear magnetic resonance (NMR) spectrometer.
10. Plate reader equipped with fluorescence polarization filter set (Ex/Em = 490 nm/520 nm)

3. Methods

All methods done with commercial kits are conducted using recommended manufacturers' protocols.

All glassware and pipette tips should be sterilized before use.

3.1 Preparation of the Cyclic Peptide Library

The schematic construct of phagemid library is illustrated in Figure 1c.

1. Use polymerase chain reaction method to construct the phagemid library. The pADL10b vector was amplified with two primers, pADL-F: 5'- GGT CCG TCC ATG GCC TGC NNK NNK NNK NNK NNK TAG GGC CCG GG-3' and pADL-R: 5'-CCA CGG CCA TGG CCG GCT GGG CCG CG-3'. The pADL-F contains six randomized coding sites as the library. (see Note 1)
2. Digest the PCR products with DpnI, and then purify the digested DNA samples with QIAquick PCR purification kit.
3. Digest the products from previous step with NcoI-HF, and then purify the reaction mixtures by agarose gel electrophoresis using QIAquick gel extraction kit.
4. Use T4 ligase to ligate digested products, purify the reaction mixtures with QIAquick PCR purification kit, and quantify the purified DNA solution.
5. Perform 10 electroporations with Top10 electrocompetent cells. Prechill 2mm electroporation cuvettes, DNA library solutions, and 0.6 ml Eppendorf tubes. For each transformation, mix 600 ng of DNA with 240 uL of Top10 cells in a 0.6 ml Eppendorf tube. Transfer the mixture to an electroporation cuvette, and electroporate using the MicroPulser system with the pre-programmed setting, Ec2. Then immediately add 2 ml of 2YT medium to the cuvette, and transfer the mixture to a 5 ml culture tube to allow the cells to recover for 45 min at 37 °C. (see Note 2)
6. Combine all cell cultures, then transfer 100 uL of the cell culture into a 1.6 mL Eppendorf tube. Perform ten-fold serial dilutions by mixing 100 uL of the cell culture with 900 uL of 2YT medium. After dilution, take 100 uL out of 10^{-5} and 10^{-6} dilutions and plate them on LB agar plates supplemented with ampicillin, and then allow the plate to grow over night. Meanwhile inoculate the rest of the cells into 1 L of 2YT medium with ampicillin. The next day, calculate the transformation yield and coverage of the library by counting the numbers of colonies on plates. (see Note 3)

¹Use the conditions recommended by Phusion® High-Fidelity Polymerase PCR Kit protocol to determine reagent usage. For thermocycles, allow annealing at 58 °C for 30 sec and extension at 72 °C for 4 min.

²Prechill electroporation cuvettes on ice for at least 30 min before the experiment. During mixing and transferring process, do them gently to prevent cell disruption. Make sure to get rid of moisture from the cuvette right before electroporation to prevent arcing. Prewarm 2YT medium and add it immediately to the mixture after electroporation generally helps the transformation efficiency.

³To calculate the library coverage, count the number of colonies on the plate and use the following equation to convert it into number of transformants. For example, if 10 colonies are observed on the plate made from 10^{-6} dilution solution, the number of total transformants acquired from the electroporations equal to: $10 \text{ (colony number)} \times 10^6 \text{ (dilution factor)} \times 20 \text{ mL (total volume)}/0.1 \text{ mL (volume used for plating)} = 2 \times 10^9$.

7. Once a good coverage of the library is confirmed by plates. Extract the library phagemids from the 1 L cell culture using a Midiprep kit. (see Note 4)
8. Prepare Top10 cells containing M13KO7TAA and pEVOL-PrKRS-CloDF plasmids [11] (hereafter Top10*) by co-transforming two plasmids into chemical competent Top10 cells with standard heat shock protocols. (see Note 5)
9. Grow the Top10* cells in 1 L of no salt LB medium supplemented with chloramphenicol and kanamycin to OD600 0.4. Pellet the cells (4000 rcf, 5 min), then wash the pellet with 10% cold glycerol. Repeat the glycerol wash 2 more times to ensure the competency of the cells.
10. Perform 10 electroporations with the extracted phagemid DNA from step 3.1.7 and electrocompetent Top10* cells from step 3.1.9 using the method mentioned in step 3.1.5 - 3.1.6. Use ampicillin, chloramphenicol, and kanamycin for all the plates and media involved.
11. Once a good coverage of the library is confirmed by plates, pellet 1 L of the transformed Top10* cells (4000 rcf, 15 min), and then resuspend the pellet with 100 mL of 20% glycerol in 2YT medium. Aliquot and store the cell stocks in -80°C .

3.2 Expression of the Cyclic Peptide Library

1. Grow the cells from step 3.1.11 in 1 L 2YT medium, containing ampicillin, chloramphenicol, and kanamycin to OD600 0.5. Induce phage expression by adding 0.2% arabinose, 1 mM IPTG, and 5 mM AcrK [11] to the cell culture, then incubate the mixture at 30°C for 16 h.
2. Centrifuge the cell culture (4000 rcf, 15 min) and collect the supernatant. Precipitate phages by adding the 5X precipitation solution to the supernatant, then allow the mixture to sit on ice for 2 h.
3. Centrifuge the mixture (10000 rcf, 30 min), remove the supernatant, and re-dissolve the phage pellet in 1 ml PBS buffer.
4. Remove cells by centrifugation (4000 rcf, 15 min) and collect the supernatant.
5. Heat the phage solution to 65°C for 15 min to kill all the remaining cells.
6. Meanwhile, grow a 5 ml Top10F' cell culture to OD600 0.5 with tetracycline. Take 10 μL of phage solution from step 5 to perform ten-fold serial dilutions with 90 μL of PBS buffer.
7. Mix 10 μL of the diluted solutions to 90 μL of Top10F' cells, and then incubate the mixtures at 37°C for 45 min.

⁴Since the cyclic peptide library contains 6 randomized coding sites, the size of the library should be $20^6 = 6.4 \times 10^7$. An appropriate library coverage should be at least 10 times higher than the library size. Meaning the minimum transformants obtained from the electroporations should exceed 6.4×10^8 .

⁵M13KO7TAA is helper phage plasmid derived from M13KO7 with a single TAA mutation at the K10 coding site of the gIII gene. pEVOL-PrKRS-CloDF is a plasmid encoding a t-RNA synthetase for genetic code expansion. The plasmid is derived from pEVOL-PrKRS [12] with a CloDF replacing the p15a replication origin in pEVOL-PrKRS for a better compatibility with pADL expression system. The M13KO7TAA and pEVOL-PrKRS-CloDF confer kanamycin and chloramphenicol resistance, respectively.

8. Spot 10 uL of the mixtures on a LB agar plate with ampicillin and tetracycline, and then incubate the plate at 37 °C overnight.

9. Calculate the yield and the coverage of library by counting plaques formed on the plate. (see Note 6) The resulted solution from step 3.2.5 contains a cyclic peptide library that can be used to screen out binders for protein targets. For the following protocols, the HDAC8 [11] is used for demonstration.

3.3 Panning Against HDAC8 Using Streptavidin Magnetic Beads

1. Transfer 100 uL of streptavidin magnetic bead slurry to a 1.6 mL Eppendorf tube. Wash the beads three times with 1 mL PBS buffer, and resuspend beads in 100 uL PBS, then split into two tubes.
2. Add 10 ug of biotinylated HDAC8 [11] in 500 uL PBS buffer to one of the tubes (hereafter +HDAC8 tube), and just 500 uL of PBS buffer to the other (hereafter -HDAC8 tube). Let the mixtures incubate at room temperature for 30 min.
3. Remove the supernatants from both tubes, wash the beads with 1 mL PBS buffer three times, and then resuspend the beads in 1 mL 1X blocking buffer. At the same time, add 0.25 mL of 5X blocking buffer to the phage solution from step 3.2.5, and incubate all three tubes at room temperature for 30 min.
4. Remove the blocking buffer from the -HDAC8 tube, transfer the phage solution into this tube, and then incubate the mixture at room temperature for 30 min as the negative selection.
5. Remove the blocking buffer from the +HDAC8 tube, transfer the phage solution from -HDAC8 tube to +HDAC8 tube, and then incubate the mixture at room temperature for 30 min as the positive selection.
6. After positive selection, remove the phage library, and wash the resins 4 times with 1 mL wash buffer.
7. Elute the bound phages by incubating the beads with 100 uL of glycine solution for 15 min.
8. Use the magnetic rack to separate the solution from the beads. Transfer the solution to a new 1.6 mL Eppendorf tube, and then neutralize it with 50 uL Tris solution.
9. Take 10 uL out of 150 uL neutralized phage solution to perform ten-fold serial dilutions with PBS buffer.

⁶.To estimate the phage expression yield, count the plaques formed on the plate and use the following equation to convert it into the number of total virions. For example, if 10 plaques are observed on 10^{-4} dilution spot, the number of total infective virions acquired from the expression equal to: $10 \text{ (plaque number)} \times 10^4 \text{ (dilution factor from serial dilution)} \times 10 \text{ (dilution factor from mixing with cells)} \times 1 \text{ mL (total phage solution volume)} / 0.01 \text{ mL (volume used for titering)} = 10^8 \text{ pfu}$. To ensure a good coverage of library, the expression yield should be at least 10 times higher than the library size. Meaning the minimum virions obtained from the expression should exceed 6.4×10^8 .

10. Meanwhile, grow a 25 ml Top10F⁺ cell culture to OD₆₀₀ 0.5 with tetracycline, and then use the diluted solutions from step 3.3.9 to perform titer assay as described in step 5.2.6 - 5.2.9. (see Note 7)
11. Mix the rest 140 μ L phage solution with the remaining Top10F⁺ cell culture, and incubate the mixture at 37 °C for 45 min.
12. Centrifuge the culture (4000 rcf, 15 min), resuspend the pellet in 400 mL 2YT medium supplemented with ampicillin and tetracycline, and then allow cells to amplify at 37 °C overnight.
13. Extract the library phagemid from the overnight culture using a Miniprep kit.
14. Perform 1 electroporation with the extracted phagemids into Top10* electrocompetent cells using the method described in step 3.1.10 - 3.1.11.
15. Use the transformed Top10* cells and repeat steps in section 3.2 to express phages for the following round of selection.
16. Repeat the selection process three more times to enrich consensus sequences.
17. After the fourth round of selection, pick 20 colonies from the plate used in the titer assay and sequence them to identify enriched sequences. For the following protocols, an octapeptide Cych8a identified from the HDAC8 selection was synthesized and assayed.

3.4 Synthesis of 5-FAM Conjugated Cyclic Octapeptide Cych8a

Figure 1 outlines the overall procedure and details for the solid-phase peptide synthesis of 5-FAM conjugated Cych8a

1. Transfer 200 mg Rink amide resin in DMF to a 50 mL SPPS vessel. Allow the mixture to sit at room temperature for 1 h.
2. Remove DMF using vacuum filtration.
3. Transfer 10 mL 20% (v/v) piperidine/DMF solution into the peptide synthesis vessel, and let the mixture react for 30 min.
4. Drain the mixture, and wash the resins with DMF, DCM and methanol.
5. Dissolve Fmoc-Lys(Mtt)-OH (4 eq.), HBTU (4 eq.), and DIEA (10 eq.) in 5 mL DMF and then add this solution to the reaction vessel under nitrogen to mix with the resin.
6. Determine the completion of coupling using the Kaiser-ninhydrin test. (see Note 8)

⁷.Top10F⁺ is a strain of *E. coli* that contains F pili and can be infected by M13 phages. The F⁺ episome carries the tetracycline resistance gene.

⁸.The Kaiser-ninhydrin test can be used to monitor the completion of a coupling reaction. Transfer a small amount of resin to a test tube and add 2-3 drops of reagent A-C, sequentially. Heat the mixture to 100 °C for 5 minutes, and check the color change. A yellow color suggests the absence of free amine, meaning the coupling is finished, while a purple indicates the presence of free amine and therefore the coupling is not complete.

7. Once the Kaiser-ninhydrin test shows negative result, add 5 mL of 1% TFA and 5% TIS in DCM (v/v) to the resin to deprotect Mtt group. Check the reaction with Kaiser-ninhydrin test.
8. Add 5-FAM (2 eq.) and DIEA (5 eq.) in 5 mL DMF to the resin, and then let the reaction proceed until the Kaiser-ninhydrin test becomes negative.
9. Repeat deprotection and coupling as described in step 3.4.3-3.4.5 accordingly to couple sequentially with Fmoc-Lys(Mtt)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Met-OH, Fmoc-Trp(Boc)-OH, Fmoc-Leu-OH, Fmoc-Ser(OtBu)-OH, and Fmoc-Cys(Trt)-OH.
10. Repeat step 3.4.7 to remove Mtt group from the second lysine. Check the reaction with Kaiser-ninhydrin test.
11. Add *N*-Succinimidyl acrylate (2 eq.) and DIEA (5 eq.) in 5 mL DMF to the resin and carry out the coupling until Kaiser test becomes negative.
12. Deprotect *N*-terminal Fmoc group using the method described in step 3.5.3.
13. Deprotect the side chain protecting groups and cleave the peptide from the resin by adding 4 mL of cleavage cocktail containing 92.5% TFA, 2.5% TIS, 2.5% water, 2.5% DODT. Let the mixture sit at room temperature for 4 h, and then precipitate the peptide with 40 mL of cold diethyl ether and collect the peptide by centrifugation (4000 rcf, 15 min).
14. Purify the peptide with HPLC and lyophilize the purified product. (see Note 9)
15. To generate cyclic peptide, dissolve peptide in PBS buffer at room temperature for 4 h.
16. Purify the cyclized peptide with HPLC using the same condition in step 3.4.13 and lyophilize the purified product. Check the cyclization using NMR analysis to make sure no peaks of alkenyl hydrogens are presented in the spectrum.

3.5 Fluorescence polarization measurement

1. Dissolve 5-FAM conjugated Cych8a in DMF to prepare a 100 μ M stock solution.
2. Serial dilute HDAC8 protein sample with 0.1% Tween20 in PBS buffer to prepare 200 μ L of solutions with concentrations ranging from 160 nM to 160 μ M.
3. Add 0.5 μ L of peptide stock solution to each diluted protein solutions, transfer the mixtures to a 96-well plate, and incubate the peptide/protein mixtures at 37 $^{\circ}$ C for 30 min.
4. Measure the fluorescence polarization in a microplate reader using filter set: Ex/Em = 490 nm/520 nm

⁹The crude Cych8a was first dissolved in 1 mL of DMF, and then subjected to a semi-preparative HPLC equipped with a Discovery BIO wide pore C18-5 column (25 cm x 10 mm). Gradient conditions: 0-50% acetonitrile over 30 min, flow rate: 4 mL/min

3.6 IC50 value measurement

1. Dissolve 5-FAM conjugated CycH8a in 10% DMF in PBS buffer to prepare 20 mM peptide stock solution.
2. Dilute peptide stock solution 10 times with PBS buffer to make a 2 mM peptide solution and bring down DMF content to 1%.
3. Serial dilute the peptide solution with 1% DMF in PBS buffer to make 200 uL of solutions with concentrations ranging from 1 nM to 1 mM.
4. Prepare 2 μ M HDAC8 solution with 1% DMF in PBS buffer.
5. Add 0.5 uL of HDAC8 solution to each diluted peptide solutions, transfer the mixtures to a 96-well plate, and incubate the peptide/protein mixtures at 30 °C for 10 min.
6. Add Boc-Lys(Ac)-AMC to each well to a final concentration of 50 μ M, and incubate the mixture at 30 °C for 1 h.
7. Add 1 μ M of trichostatin A and trypsin (0.5 mg/ml) to the reaction to quench the reaction, and incubate the mixture at 30 °C for 1h.
8. Measure the fluorescence of coumarin in a microplate reader with Ex/Em = 360 nm/460 nm.

Acknowledgments

This work was supported in part by National Institute of Health (grant R01CA161158), Cancer Prevention and Research Institute of Texas (grant RP170797), and Welch Foundation (grant A-1715).

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Fig. 1.

(a) A proposed proximity-driven cyclization between a cysteine and an electrophilic noncanonical amino acid (ncAA), AcrK. (b) An amber-codon suppression-based approach to link the phenotypic ncAA with the genotypic TAG mutation. A phage with a TAG mutation at the coding region of its displayed peptide is produced in *E. coli* that harbors an evolved aminoacyl-tRNA synthetase and amber-suppressing tRNA for the genetic incorporation of the designated ncAA. (c) Schematic construct for production of a phage-displayed 6-mer cyclic-peptide library.

Fig. 2.

Solid-phase peptide synthesis of 5-FAM conjugated Cych8a. Reagents and conditions: (i) 20% piperidine in DMF, 30 min; (ii) Fmoc-Lys(Mtt)-OH, HBTU, DIEA, DMF, 4 h; (iii) TFA/TIS/DCM 1:5:94, 30 min; (iv) 5-FAM, HBTU, DIEA, DMF, 4 h; (v) Fmoc-AA-OH, HBTU, DIEA, DMF, 4 h; (vi) *N*-succinimidyl acrylate, HBTU, DIEA, DMF, 4 h; (vii) TFA/TIPS/H₂O/DODT 92.5:2.5:2.5:2.5, 4 h; (viii) PBS buffer pH7.4, 4h.