Supporting Information

for

In vitro Selection of a Photo-responsive Peptide Aptamer by Ribosome Display in the Presence of Azobenzoyl Lysine-tRNA

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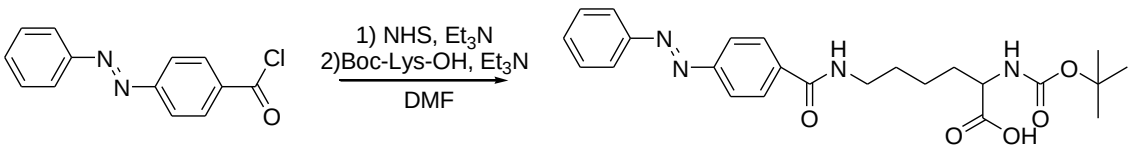
1. Materials and Methods

1.1. Synthesis of azobenzene-lysine

tBoc-*ε*-(azobenzoyl)-lysine was synthesized as shown in Scheme S1. 4-Phenylazobenzoyl chloride (1.08 g, 4.09 mmol) was dissolved in 20 ml of N, N-dimethylformamide (DMF), and N-hydroxysuccinimide (NHS) (518 mg, 4.5 mmol) and Triethylamine (Et_3N) (0.86 mL, 6.13 ml) were added to the solution at 4 °C. The mixed solution was stirred for 10 min. Subsequently, tBoc-lysine (1.01 g, 4.09 mmol) and Et_3N (0.57 mL, 4.09 mmol) were added to the solution and left at room temperature. After evaporation, the product was extracted with ethyl acetate (EtOAc), washed with an aqueous solution of 10% citric acid three times and dried with $NaSO_4$. The product was referred to as tBoc-*ε*-(azobenzoyl)-lysine. 1H-NMR (400 MHz, DMSO-d6): δ 8.70–8.67 (t, 1H, J = 5.6), 8.08–8.06 (d, 2H, J = 8.4), 7.96–7.92 (m, 4H), 7.65–7.58 (m, 3H), 6.71–6.69 (d, 1H, J = 7.6), 3.82–3.77 (t, 1H, J = 8.0), 3.30–3.25 (m, 2H), 1.72–1.51 (m, 4H), 1.37 (s, 9H) and 1.37–1.30 (m, 2H). HR-TOF-MS m/z: calculated for $C24H30N4O5Na^+$ ($[M+Na]^+$) 477.2114; Observed 477.2104.



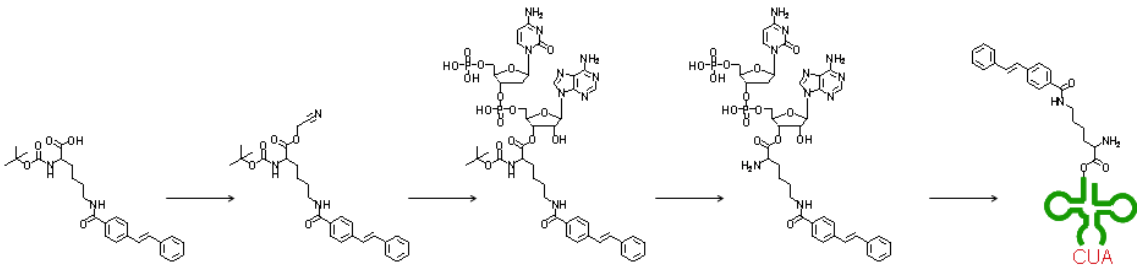
Scheme S1

1.2 Preparation of azobenzoyl-lysine-tRNA

The preparation scheme is shown in Scheme S2. Azobenzoyl-lysine-pdCpA was synthesized as follows. To a mixture of tBoc-*ε*-(azobenzoyl)-lysine (50 mg, 0.11 mmol) and triethylamine (36 μL) in acetonitrile (0.6 mL), chloroacetonitrile (12 μL) was gradually added on ice. The mixture was stirred at room temperature for 18 h, acidified to pH 2 with aqueous 5% $KHSO_4$ and extracted with ethyl acetate. The extract was washed three times with aqueous 5% $KHSO_4$, three times with aqueous 4% $NaHCO_3$, once with saturated aqueous NaCl and dried over sodium sulfate. The solvent was evaporated to give the tBoc-*ε*-(azobenzoyl)-lysine cyanomethyl ester. Acylation of 5'-O-phosphoryl-2'-deoxycytidylyl-(3'-5')adenosine (pdCpA) was carried out by adding tBoc-*ε*-(azobenzoyl)-lysine cyanomethyl ester (44 μmol) to a DMF solution of the pdCpA tetrabutylammonium salt (75 μL, 3.3 μmol). The resulting solution was incubated at 37 °C for 1 h. Diethyl ether (600 μL) was added to the solution and the

precipitate was collected by centrifugation. The resulting precipitate was washed twice with diethyl ether and dried under vacuum. The pellet was dissolved in trifluoroacetic acid (200 μL) and placed on ice for 15 min to remove the Boc-group. After evaporating trifluoroacetic acid by vacuum centrifugation, the pellet was washed twice with diethyl ether (600 μL) and dried under vacuum. The product was confirmed by MALDI-TOFMS (Voyager, Applied Biosystems: calculated for (M-H)-971.26, found 971.22).

The resulting azobenzoyl-lysine-pdCpA was ligated to an amber suppressor tRNA derived from *Mycoplasma capricolum* Trp_1 tRNA without the 3' dinucleotide by a chemical ligation method described previously.[1,2] The azobenzoyl-lysine-tRNA can now be obtained as a commercially available reagent (CoverDirect tRNA reagents for site-directed protein labeling, ProteinExpress, Chiba, Japan).



Scheme S2. Synthetic route of Azobenzoyl-lysine-tRNA.

1.3 *In vitro* selection

The protocol for the ribosome display that we developed in the present investigation is shown in **Scheme 1**. The DNA template for *in vitro* selection was similar to that reported previously[3-5] and is shown in **Figure S1**. The sequence comprised: a T7 promoter (5'-TAATACGACTCACTATA-3') for *in vitro* transcription; SD, a Shine–Dalgarno *E. coli* ribosome-binding site sequence; random sequence region; Trx, the gene for thioredoxin; and Ps, the gene for the protein spacer derived from dihydrofolate reductase.

Scheme S3

First, a single-stranded oligonucleotide (ssDNA) was prepared to construct the

sequence encoding the random sequence peptide library. The ssDNAs, 5'-ATATGGCCATGCAGGCCTAG $(NNN)_3$STOP$(NNN)_7$ GGCCAGCTAGGCCAGTT-3' and 5'-ATATGGCCATGCAGGCCTAG $(NNN)_7$STOP$(NNN)_3$ GGCCAGCTAGGCCAGTT-3'where N = G, C, T, or A, were synthesized using a solid-phase method, and double-stranded oligonucleotides (dsDNA) were prepared using DNA polymerase in the presence of the reverse primer rp-2 (5'-AACTGGCCTAGCTGGCC-3'). The resulting dsDNA products were digested by *SfiI* and purified by polyacrylamide gel electrophoresis (PAGE). The purified fragments were then inserted between the *SfiI* sites located in the plasmid. Finally, DNA templates for in vitro selection were prepared by PCR using the ligated product and the primers fp-1 (5'-TTAATACGACTCACTATAGAACATGAGGATCACCCATGTAAAAGTCGACAA TAATTTTGTTTAACTT-3') and rp-1 (5'- AAACAGCTATGACCATGATTA-3'). The prepared DNA template was used for *in vitro* transcription and translation. The transcription was performed using RiboMAX Large Scale RNA Production Systems (Promega). 10 µL of 5× Transcription Buffer (400 mM HEPES-KOH pH 7.5, 120 mM $MgCl_2$, 10 mM spermidine, 200 mM DTT), 5 µL of T7 RNA Polymerase, 2.5 µL of rNTP (100 mM each), RNase inhibitor (2 U) and 10 µL of template DNA (1 µg) were mixed, and the solution was diluted with ultra-pure water to a final volume of 50 µL, and incubated at 37 °C for 3 h, after which the reaction was stopped by adding 2.5 µL of DNase. The sample was incubated for another 1 h. The mRNA was purified using an RNeasy® Mini kit purchased from Qiagen (Hilden, Germany) according to the manufacturer's manual. The yield was confirmed by 2% agarose gel electrophoresis and the concentration of the mRNA was measured by ultraviolet (UV) absorbance.

In vitro translation was performed using the PURESYSTEM 1 RF1 in the presence of prepared AZ-lysine-tRNA. Thirty-five microliters of the translation kit, mRNA (4 pmol), RNase inhibitor (2 U) and AZ-tRNA (320 pmol) were mixed, and the mixture was diluted with ultra-pure water to a final volume of 50 µL and left at 37 °C for 20 min. After the reaction was stopped by placing the mixture on ice for 10 min, 250 µL of Selection buffer 1 (60 mM Tris-acetate, pH 7.5, 180 mM NaCl, 0.06% Tween 20, 60 mM $Mg(AcO)_2$) was added.

Streptavidin-immobilized magnetic beads (Dynabeads M-280 streptavidin) were purchased from Veritas Co. (Tokyo, Japan) and used in the experiments according to the manufacturer's protocol. The target-immobilized microbeads (10 µL, 3.5–6 × 10^7) were incubated with the translated solution and 250 µL of the selection buffer (60 mM Tris-acetate pH 7.5, 180 mM NaCl and 60 mM magnesium acetate) at 4 °C for 1 h. The

beads were recovered with a magnet and washed with washing buffer (200 µL, 50 mM Tris-acetate pH 7.5, 150 mM NaCl, 0.05% Tween 20; and 50 mM magnesium acetate) eight times at 4 °C. Finally, 50 µL of the washing buffer was added to the dried beads and the mixture was irradiated with UV light (HAMAMATSU, UV spot light source) through a glass filter (Asahi Techno Glass, UV-D36B) at 4 °C for 2 min. The beads were separated from the solution with a magnet and solvent was collected. mRNA was recovered from the solution by 2× Elution buffer (100 mM Tris-acetate, pH 7.5, 300 mM NaCl, 0.1% Tween 20, 120 mM EDTA) at 4 °C for 2 h. The isolated mRNA was purified using a RNeasy mini kit (Qiagen, Germany).

The isolated and purified mRNA template was mixed with 20 □L of rp-fp-M13-NS primer (1 µM), 5 µL of dNTP Mix (10 mM each) and a ribonuclease inhibitor in a nuclease-free tube, and the mixture was diluted with ultra-pure water to a final volume of 75 µL and incubated at 70 °C for 5 min. The mixture was cooled immediately on ice for 5 min and the reaction mixture was prepared by combining with the reverse transcriptase solution (20 µL of 5× PrimeScript Buffer (250 mM Tris-HCl pH8.3, 375 mM KCl, 15 mM MgCl2), 1 µL of RNasin (40 U) and 4 µL of PrimeScript Reverse Trascriptase (800 U)). The reaction mixture was pipetted gently and incubated at 50 °C for 1 h. The solution was then incubated at 70 °C for 10 min and cooled immediately on ice for 5 min.

Preparative PCR was performed to amplify the reverse transcription products using primers T7-fp-rec-1 and rp-fp-M13-NS. Ten microliters of 5× PrimeSTAR GXL Buffer, 1 µL of PrimeSTAR GXL DNA Polymerase (1.25 U), 4 µL of dNTP (2.5 mM each), 10 µL of T7-fp-rec-1 primer (1 µM) and 10 µL of rp-fp-M13-NS primer (1 µM) were mixed, and the solution was diluted with ultra-pure water to a final volume of 50 µL and amplified using 18 cycles (98 °C, 10 s; 55 °C, 15 s; 68 °C, 60 s). The DNA product was isolated using the QIAGEN PCR Purification kit (Qiagen, Germany), the quality of the PCR product was verified by analysis on 6% PAGE and its concentration was measured by UV absorbance. The isolated DNA was used directly as the template for the next round of selection.

1.4 Subcloning and Sequencing

After five rounds of these processes, the sequences of the selected DNAs were analyzed according to the protocol shown in Figure S5. The recovered DNA was amplified using PCR with primers fp-Lib-pUXS2 and rp-Lib-pUXS2. The DNA product was purified using a purification kit, the quality of the PCR product was verified by analysis on a 5% PAGE and its concentration was measured by UV absorbance. The

recovered DNA and vector fragment were treated with *SfiI* at 50 °C overnight, the digested DNA fragment was inserted into the vector fragment by a ligation reaction (DNA Ligation kit <Mighty Mix>) and the mixture was incubated at 16 °C overnight. The ligated product was transformed into *E. coli*, and the transformed individual clones were sequenced at the RIKEN BSI Research Source Center.

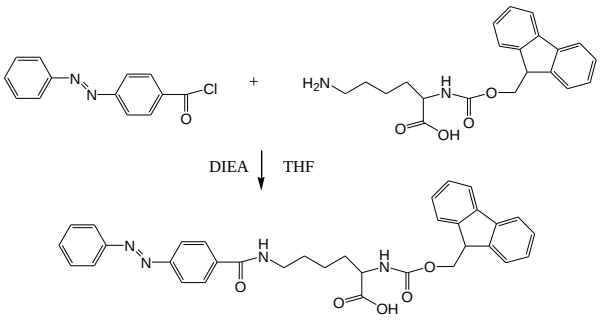
1.5 Primer sequences

T7-fp-rec-1 primer
5'-TTAATACGACTCACTATAGAAAAGTCGACAATAATTTTGTTTAACTT-3'
rp-fp-M13-NS primer
5'-AAACAGCTATGACCATGATTA-3'
fp-Lib-pUXS2 primer
5'-ATATGGCCATGCAGGCC-3'
rp-Lib-pUXS2 primer
5'-AACTGGCCTAGCTGGCC-3'

1.6 Chemical synthesis of the peptide

1.6.1 Synthesis of Fmoc-azobenzoyl Lys-OH（Fmoc-AZ-lysine-OH）

Fmoc-azobenzoyl Lys-OH was prepared as shown in Scheme S4. Fmoc-Lys-OH (2.21 g, 6.0 mmol) was dissolved in 40 mL of dried tetrahydrofuran (THF). To the solution 4-phenylazobenzoyl chloride (1.47 g, 6.0 mmol) and N,N-diisopropylethylamine (DIEA, 1.14 mL, 2.2 mmol) were added at 4 °C and the solution was stirred for 1 h. Subsequently the solution was left for another 11 h at room temperature. After filtration and evaporation, the residue was collected and dissolved with EtOAc. The solution was washed with distilled water twice and with NaCl saturated water twice and finally dried with $NaSO_4$. By column purification, Fmoc-AZ-lysine-OH was obtained (1.86 g, 53.7 %). ^{1}H NMR 400 MHz (DMSO-d_6). δ 12.58 (s, 1H), 8.65 (s, 1H), 8.03 (s, 2H), 7.92–7.86 (m, 8H), 7.70 (s, 2H), 7.60 (s, 4H), 7.39–7.30 (m, 5H), 4.25 (d, 2H, J = 4.00), 4.22 (s, 1H), 4.20 (s, 1H), 3.28 (m, 2H), 1.37 (br, 4H). Hi-Mass (576.23), observed 599.22 ($M + Na^+$).



Scheme S4. Synthesis of Fmoc-AZ-lysine-OH.

1.6.2 Solid phase synthesis of the peptide containing azobenzene

Fmoc-AZ-lysine-OH was incorporated into the general Fmoc-based peptide synthesis using a solid phase method at the RIKEN BSI Research Bioresource Center.

1.7 Binding assay

The peptide (1 μM) and streptavidin-immobilized microbeads (3 μL, 1.2–2.0 × 10^7) were suspended in 500 μL of washing buffer (50 mM Tris-acetate pH 7.5, 150 mM NaCl, 0.05% Tween 20, 50 mM $Mg(AcO)_2$, 1% DMSO), and then incubated at 20 °C for 1 h. The reaction buffer containing free peptide was removed by three times-washing with washing buffer. The recovered microbeads were re-suspended in 200 μL of washing buffer. Subsequently, 200 μL of TBS-T buffer (20 mM Tris, 137 mM NaCl, 0.1% Tween20, pH 7.6) containing 10 μg/mL of the anti-FLAG antibody labeled with FITC was added, and then the solution was incubated at 20 °C for 1 h. After three times-washing with TBS-T buffer, the microbeads were re-suspended in TBS-T buffer, and then the fluorescent intensity was measured by a microplate reader. When irradiation experiments were performed, the suspension was separated into two 100 μL solutions, and either visible light or UV light irradiation was carried out on each solution.

2. Figure S1

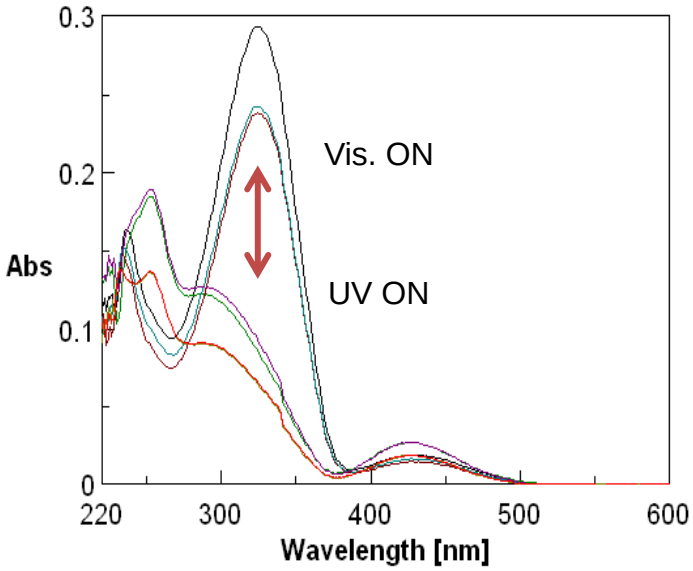


Figure S1. UV spectra of tBoc-ε-(azobenzoyl)-lysine under irradiation of visible and ultraviolet light.

3. Table S1

Table S1: Peptide sequences found by current selection. X indicates □-azozenzoyl lysine

No.	Sequences	No.	Sequences
LA01	AIHIMPWXSGM	LA53	VGAHCRVXVFE
LA02	TIGSHAIXPPL	LA54	VGVXISVSYSR
LA04	THSXDSYWGSY	LA55	EAYXWVGYRIL
LA06	RFRPNHDXCFV	LA56	LVRLVPTXVVR
LA08	TVAXMTNAVYP	LA57	LNYXDVALSTL
LA09	EIVVEWDXTDG	LA58	RGVXWGDSSYS
LA12	TRXXAIIFCYL	LA59	RCGXYACIIRV
LA13	LCPXMKYNXFW	LA60	QYLXHIPSPAA
LA14	LGHXDSNYRHN	LA61	KMGIGAYXNAI
LA15	VSDIINTXEWV	LA62	ERSVFSSXNVM
LA16	VFGXYEIRILW	LA63	VASLGFGXVFV
LA17	YRAMVARXNAF	LA64	NCDXSVAMWDV
LA18	YSSALPCXSFV	LA65	VDCVFTMXAGW
LA19	YPATNANXCXI	LA66	WSSXFLPTYLP
LA21	WGTHASAXMIF	LA67	ISRLGAGXLNF
LA22	STMTGLRXIWW	LA68	FSSXSRRGNVA
LA23	DDSLVGRXFSP	LA69	VAIXGYALEAV
LA24	SRTDWGHXARV	LA70	QWTXMLNNCND
LA25	LNAXMDVFGIL	LA71	CYWXAHGISLF
LA26	VMSXIALPVLL	LA72	NPVYLLGXLKW
LA28	SPMXCFRHMTP	LA73	CPIWSGIXSWP
LA29	SWYFVSEXNEW	LA75	LAYWSAVXKIP
LA30	IAERQAHXVDM	LA77	CRFKFVVXAGS
LA32	LYIXQGTXSCV	LA78	SASCLXLXRSR
LA33	HSATAQMXIGN	LA79	VGLSSCPXDAF
LA34	TFSXDSLAGFL	LA80	VIHXASFHPNM
LA35	IXHXGEFPPAS	**LA81**	**GVTXRRFIXYV**
LA37	VLIMVAVXASS	LA82	ARTXGALYCWX
LA38	EYXXIPAHLSP	LA83	VGGXSHWFIGF
LA39	LLTVMAPXRIV	LA86	LYGGMCGXLRF
LA40	HSCXVTIDVFF	LA87	IRRGVGTXVAI
LA41	SXQXGAFVNGG	LA88	HATXVCTGYVW
LA42	LNVCAGAXGVV	LA89	PNTXYCNRVYD
LA43	HSCXVTIDVFF	LA90	RVVXILGPVIC
LA44	RLNLFASXGDY	**LA91**	VLIMVAVXASS
LA45	ACLCQEVXPLH	LA92	NALITTMXGWA
LA47	VHLXTIMSEGV	**LA93**	HSCXVTIDVFF
LA48	VLIMVAVXASS	LA94	LKLQPESXYLF
LA49	FGFDLAFXKWW	LA95	TWWARHLXSDY
LA50	VIVCMSRXRTG	LA96	RIVXXCFIRPM
LA51	TYLRMRLXVIW		

4. References

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