ChemComm

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Journal:	*ChemComm*
Manuscript ID	CC-COM-01-2022-000584.R2
Article Type:	Communication

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In vitro display evolution of unnatural peptides spontaneously cyclized via intramolecular nucleophilic aromatic substitutions

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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We report novel, ribosomally incorporatable, and intramolecularly cysteine-reactive fluorobenzoic acid-derived linkers for SELEX of mRNA-displayed unnatural peptides, which spontaneously cyclized via intramolecular nucleophilic aromatic substitutions forming thioethers. With this strategy we identified several novel PCSK9-binding peptides.

Cyclic peptides have recently attracted attention as a novel drug modality to solve the problems of small molecules and antibodies as existing pharmaceutical products. Cyclic structures of peptides can not only increase their conformational rigidity but also give different conformations of the peptides with their different linkages. Thus, the development of a platform that can incorporate various cyclic structures into peptide libraries applicable to high throughput screening is highly important for peptide drug discovery.

Hypercholesterolemia, which is caused by excessive accumulation of low-density lipoprotein cholesterol (LDL-C) in plasma, increases the risk of atherosclerosis, potentially leading to the development of cardiovascular diseases.[1-3] Proprotein convertase subtilisin/kexin type 9 (PCSK9) interacts with the LDL receptor (LDLR), which is responsible for the uptake of plasma LDL-C into hepatocytes, and induces its lysosomal degradation.[2-4] Therefore, PCSK9 is an attractive therapeutic target for the treatment of hypercholesterolemia.

The primary treatment for hypercholesterolemia at present is the administration of small molecule statins that promote the expression of LDLR.[5] However, because statins also promote the PCSK9 expression, a combination of statins and anti-PCSK9 antibodies, such as alirocumab and evolocumab, has also been used in clinical settings, for the treatment of hypercholesterolemia.[6-8] Camel-derived anti-PCSK9 single-domain antibodies,[9] anti-PCSK9 fragment of antigen-binding, PCSK9-binding engineered fibronectin, and LDLR fragments have been developed as alternatives to anti-PCSK9 antibodies. However, these macromolecular PCSK9-binding proteins have some disadvantages, including high costs because of production in cell culture, immunogenicity, and oral unavailability. Small molecules generally do not effectively inhibit flat, wide-area PCSK9/LDLR interactions.[10]

Several PCSK9-binding peptides, which can be chemically synthesized at a low cost, and are potentially orally available, have been developed.[11] However, PCSK9-binding peptides derived from natural products have weak inhibitory activity. PCSK9-binding peptides designed based on the 3D structure of the PCSK9-binding site of LDLR also have been reported to have weak affinity,[1] probably since these peptides do not completely mimic the structure of the PCSK9-binding site of LDLR, and because the PCSK9/LDLR interaction is inherently not strong (K_D = 620–810 nM).[2] *De novo* PCSK9-binding linear peptides have been identified by *in vitro* selection (systematic evolution of ligands by exponential enrichment, SELEX), using phage display.[11] However, linear peptides have low peptidase resistance, due to their conformational flexibility. The size of a library available for phage display is also limited by the transformation efficiency of *Escherichia coli* (~10^9).[12] To overcome these problems, mRNA display (*in vitro* virus) has been used to discover PCSK9-binding peptides (~10^{12} in library size) cyclized by *m*-dibromoxylene linkers to have a structure more rigid than that of linear peptides.[13] However, since the *m*-dibromoxylene linker is post-translationally incorporated into the mRNA-displayed peptides, an additional cyclization process during SELEX is required.

Here we report two novel fluorobenzoic acid-derived intramolecularly cysteine-reactive linkers, which can be incorporated by ribosomes and lead to spontaneous aryl-thioether-cyclization via nucleophilic aromatic substitution forming aryl-thioether bridged peptides. The fluorobenzoic acid-derived linkers could be co-translationally incorporated into ribosomally synthesized peptides by sense codon suppression. We also found that aryl-thioether bonds can be spontaneously formed under reducing conditions, such as translation and reverse-transcription (RT), unlike disulfide bonds. mRNA-displayed peptides can therefore be cyclized

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Electronic Supplementary Information (ESI) available:
See DOI: 10.1039/x0xx00000x

without the need for an additional cyclization process during SELEX. We used a reconstituted *in vitro* transcription/translation system, Protein-synthesis Using Recombinant Elements (PURE) system without certain amino acids and aminoacyl-tRNA synthetases, allowing for efficient ribosomal incorporation of unnatural building blocks, such as fluorobenzoic acid derivatives, by sense codon suppression. Upon the removal of the competing release factors that terminate translation from the PURE system, the conjugation efficiency of the mRNA-displayed peptide at the stop codon can be also increased.

We applied two novel fluorobenzoic acid-derived linkers to SELEX of PURE system-expressed mRNA-displayed unnatural peptides cyclized via nucleophilic aromatic substitutions against the model human PCSK9 protein. We identified two novel, PCSK9-binding, unnatural cyclic peptides with completely different sequences, from two kinds of cyclic peptide libraries, demonstrating that cyclization linkers with different chemical structures confer structural diversity on cyclic peptide libraries. By applying *in vitro* affinity maturation using PURE system-expressed, mRNA-displayed focused libraries, a PCSK9-binding unnatural peptide cyclized via nucleophilic aromatic substitution was also identified using next generation sequencing (NGS). Furthermore, chemical dimers of the PCSK9-binding unnatural cyclic peptides were synthesized to increase the affinity (avidity). Finally, the chemical dimers of PCSK9-binding unnatural peptides cyclized via nucleophilic aromatic substitution were applied to PCSK9 analysis using sandwich ELISA with anti-PCSK9 antibody.

To spontaneously cyclize mRNA-displayed peptide libraries, we previously developed ribosomally incorporatable and intramolecularly cysteine-reactive chloromethylbenzoic acid and *N*-chloroacetyl amino acid derivative linkers.[12] However, we found that chloromethylbenzoic acid and *N*-chloroacetyl amino acid derivative linkers are intramolecularly not only cysteine-reactive, but also histidine-reactive, under the conditions of translation and RT used during SELEX (Fig. S1). Thus, we explored the use of less electrophilically reactive fluorobenzoic acid-derived linkers for more cysteine-specific peptide cyclization. 2-Fluoro-5-nitrobenzoic acid (FNO_2Ph) and pentafluorobenzoic acid (F_5Ph) as linkers were charged onto initiator tRNA ($tRNA^{ini}$) using tRNA-acylation ribozyme. We evaluated the translational initiation activity of FNO_2Ph-$tRNA^{ini}$ and F_5Ph-$tRNA^{ini}$, and the subsequent spontaneous peptide cyclization with downstream cysteine residues via nucleophilic aromatic substitutions, using translation into cysteine-containing model peptides by the PURE system with methionine sense codon suppression. Matrix-Assisted Laser Desorption/Ionization Time-of-Flight mass spectrometric (MALDI-TOF MS) analysis of the expressed peptides revealed that FNO_2Ph and F_5Ph could be ribosomally incorporated into peptides and spontaneously aryl-thioether-cyclize peptides under the conditions of translation and RT used in SELEX (Fig. 1 and S2). More cysteine-specific cyclization of FNO_2Ph and F_5Ph than chloromethylbenzoic acid and *N*-chloroacetyl amino acid derivatives was evaluated by translation into a histidine-containing model peptide in PURE system. MALDI-TOF MS analysis of the expressed peptides revealed that, unlike chloromethylbenzoic acid and *N*-chloroacetyl amino acid derivatives, FNO_2Ph and F_5Ph did not cyclize with downstream histidine residues under the conditions of translation and RT used in SELEX (Fig. S3). These results demonstrated that FNO_2Ph and F_5Ph are not only ribosomally incorporatable, but also more cysteine-specific spontaneous peptide cyclization linkers than previously reported chloromethylbenzoic acid and *N*-choloroacetyl amino acid derivatives.

We next applied FNO_2Ph and F_5Ph to SELEX of PURE system-expressed, mRNA-displayed unnatural peptides aryl-thioether-cyclized via nucleophilic aromatic substitutions against human PCSK9 as a model protein (Fig. 2A and S4). To prepare two mRNA-displayed peptide libraries aryl-thioether-cyclized with FNO_2Ph and F_5Ph, PCR-amplified synthetic template DNA library encoding 8–15 random amino acids between FNO_2Ph or F_5Ph cyclization linkers and downstream cysteine residues for peptide cyclization was added to release factor-free PURE system. The PURE system contained 19 proteinogenic amino acids (without Met) and FNO_2Ph-$tRNA^{ini}$ or F_5Ph-$tRNA^{ini}$, together with a puromycin-DNA linker that had a DNA sequence complementary to the 3'-untranslated region of the mRNA libraries. In the PURE system, mRNA libraries prepared by the transcription of template DNA libraries was spontaneously modified with puromycin-linker. Puromycin-modified mRNA libraries were ribosomally translated into peptide libraries initiated with FNO_2Ph or F_5Ph. Expressed peptides were spontaneously displayed on their encoding mRNAs by conjugation between the amino groups of the puromycin-linkers and the esters of the peptidyl-tRNAs at the UAG amber codon. The peptides displayed on the mRNA libraries were spontaneously cyclized via aryl-thioether bonds formed by FNO_2Ph or F_5Ph and the thiol groups of intramolecular cysteine residues in the PURE system. After RT to form double-stranded cDNA/mRNA, mRNA-displayed FNO_2Ph- and F_5Ph-cyclized peptide libraries were incubated with PCSK9-non-immobilized beads, blank streptavidin-beads to remove bead-binding peptides as negative selection. Pre-cleared mRNA-displayed FNO_2Ph- and F_5Ph-cyclized peptide libraries were then pulled down with PCSK9-immobilized beads for the isolation of PCSK9-binding FNO_2Ph- or F_5Ph-cyclized peptides as positive selection. Recovered cDNAs encoding PCSK9-binding FNO_2Ph- or F_5Ph-cyclized peptides on beads were amplified using PCR and used for the next round of SELEX. qPCR of the cDNAs showed that recovery rates of cDNAs complexed with mRNA-displayed, FNO_2Ph- or F_5Ph-cyclized peptides binding to human PCSK9-immobilized beads increased during SELEX (Fig. S5A).

PCR-amplified DNAs obtained from mRNA-displayed FNO_2Ph- and F_5Ph-cyclized peptide libraries at the final round of SELEX were cloned and sequenced (Fig. S5B). Peptide sequence alignment analysis showed that, while the FNO_2Ph-cyclized peptide library mainly converged to a peptide family containing RWRFYSGPYFILAAC and similar sequences, the F_5Ph-cyclized peptide library mainly converged to a peptide family containing RGHCWLYVYFPVRSLC and similar sequences. The differences in the converged peptide sequences between

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FNO$_2$Ph and F$_5$Ph indicated that cyclization linkers with different chemical structures produce structural diversity in cyclic peptide libraries. This finding indicates the importance of developing multiple cyclization linkers for the SELEX of mRNA-displayed unnatural cyclic peptides.

We first evaluated the PCSK9-binding of cloned mRNA-displayed cyclized FNO$_2$Ph-RWRFYSGPYFILAAC peptide 1 and F$_5$Ph-RGHCWLYVYFPVRSLC peptide 2 (Fig. S6). The binding efficiency was determined using the quantification of cDNA recovered after PCSK9-pull-down of PURE system-expressed, mRNA-displayed peptides followed by qPCR. qPCR analysis indicated that the recovery rate of cyclized FNO$_2$Ph-RWRFYSGPYFILAAC and F$_5$Ph-RGHCWLYVYFPVRSLC peptides on PCSK9-immobilized beads was higher than that of PCSK9-non-immobilized beads, blank streptavidin-beads used as a control for non-specific binding. This finding confirmed that mRNA-displayed, cyclized FNO$_2$Ph-RWRFYSGPYFILAAC and F$_5$Ph-RGHCWLYVYFPVRSLC peptides bound to PCSK9 (Fig. S6A). The PCSK9-binding of mRNA-displayed cyclized FNO$_2$Ph-RWRFYSGPYFILAAC peptide 1 was peptide-dependent (Fig. S6B). The recovery rate of mRNA-displayed, cyclized F$_5$Ph-RGHCWLYVYFPVRSLC peptide 2 was higher than that of the C17S mutant, indicating that C17 contributed to the PCKS9-binding of the F$_5$Ph-RGHCWLYVYFPVRSLC peptide (Fig. S6C).

We next chemically synthesized linear FNO$_2$Ph-RWRFYSGPYFILAAC and F$_5$Ph-RGHCWLYVYFPVRSLC peptides modified with a C-terminal biotinylated lysine using Fmoc solid-phase peptide synthesis (SPPS) and aryl-thioether-cyclized these synthetic peptides with triethylamine as the base. Chemical synthesis of the desired biotinylated cyclized FNO$_2$Ph-RWRFYSGPYFILAAC and F$_5$Ph-RGHCWLYVYFPVRSLC peptides was confirmed by MALDI-TOF MS (Fig. S7), demonstrating that unnatural FNO$_2$Ph and F$_5$Ph could be incorporated into synthetic peptides using standard Fmoc SPPS with HBTU, HOBT, and DIEA. The PCSK9-binding activity of two chemically synthesized peptides was analyzed by PCSK9-pull-down and chemiluminescent detection, using horseradish peroxidase (HRP)-labeling of peptides via streptavidin (Fig. 2B and C). Chemiluminescent analysis demonstrated that chemically synthesized cyclized FNO$_2$Ph-RWRFYSGPYFILAAC peptide 1 and F$_5$Ph-RGHCWLYVYFPVRSLC peptide 2 could bind to PCSK9. PCSK9-binding of the two chemically synthesized peptides was also confirmed using peptide-pull-down and chemiluminescent detection, using HRP-labeling of PCSK9 via anti-His-tag antibody (Fig. S8). The dissociation constants (K_D) of FNO$_2$Ph-RWRFYSGPYFILAAC peptide 1 and F$_5$Ph-RGHCWLYVYFPVRSLC peptide 2 calculated from their BLI sensorgrams were 1.1 µM and 270 nM, respectively (Fig. S9). We also found that the PCSK9-binding peptides are human PCSK9-specific compared to mouse PCSK9 (Fig. S10).

We further applied FNO$_2$Ph to *in vitro* affinity maturation of the PCSK9-binding cyclic peptide using SELEX with PURE system-expressed, mRNA-displayed focused peptide libraries. Two focused mRNA libraries encoding FNO$_2$Ph-cyclized peptides with random seven amino acid residues and fixed seven amino acid residues, the same as those of the original peptide, at the N or C terminus, respectively, were used for *in*

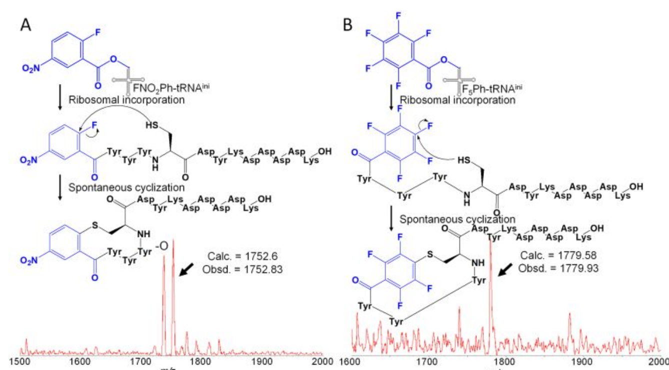

Fig. 1
(A) and (B) Translation initiation and spontaneous peptide cyclization with FNO$_2$Ph and F$_5$Ph via initiation codon suppression using PURE system; MALDI-TOF mass spectrum of FNO$_2$Ph- and F$_5$Ph-cyclized peptides. Calculated (Calc.) and Observed (Obsd.) m/z for singly charged species $[M+H]^+$ of desired peptides are shown. -O indicates nitroso-benzoic acid-cyclized peptide generated during the MALDI-TOF MS analysis.

vitro affinity maturation (Fig. S11A). Next generation sequencing analysis of the final SELEX round libraries identified the most frequent sequence as a novel sequence from the FNO$_2$Ph-cyclized C-terminal randomized library (Fig. S11B); the FNO$_2$Ph-cyclized N-terminal randomized library did not converge significantly (data not shown). A previous report indicated that the AUG codon is translated as Ile by misreading with tRNA^{Ile}, which contains a near-cognate UAU anticodon, in the Met-lacking PURE system used for SELEX.[12] Thus, a Met to Ile mutant of the most abundant sequence, FNO$_2$Ph-RWRFYSGIRNREIDC, was further assayed (Fig. S11C). The PCSK9-binding of the mRNA-displayed cyclized FNO$_2$Ph-RWRFYSGIRNREIDC peptide 3 was confirmed using PCSK9-pull-down and qPCR analysis (Fig. S12A and B). PCSK9-binding of the chemically synthesized FNO$_2$Ph-RWRFYSGIRNREIDC peptide 3 (Fig. S12C) was also confirmed using PCSK9-pull-down and HRP's chemiluminescent detection (Fig. S12D). Titration experiments indicated that the affinity-matured peptide had higher PCSK9-binding activity than the original peptide (Fig. S13). Both FNO$_2$Ph-RWRFYSGPYFILAAC peptide 1 and FNO$_2$Ph-RWRFYSGIRNREIDC peptide 3 were PCSK9-specific compared to other drug-target proteins (Fig. S14).

To improve the affinity (avidity), the PCSK9-binding FNO$_2$Ph-cyclized peptides were chemically dimerized. Linear FNO$_2$Ph-cyclized peptides dimerized with lysine linkers was chemically synthesized using Fmoc SPPS using Fmoc-Lys(Fmoc)-OH and aryl-thioether-cyclized. Chemical synthesis of the desired dimerized FNO$_2$Ph-cyclized peptides was confirmed using MALDI-TOF MS, demonstrating that unnatural FNO$_2$Ph could be incorporated into chemical peptide dimers by Fmoc SPPS. (Fig. S15). The PCSK9-binding activity of dimerized FNO$_2$Ph-cyclized peptides was compared to corresponding monomer FNO$_2$Ph-cyclized peptides using PCSK9-pull-down. Chemiluminescent analysis showed that the FNO$_2$Ph-cyclized peptide dimers had higher PCSK9-binding activity than the corresponding monomers (Fig. S16). Titration experiments indicated that the affinity-matured peptide dimer had higher

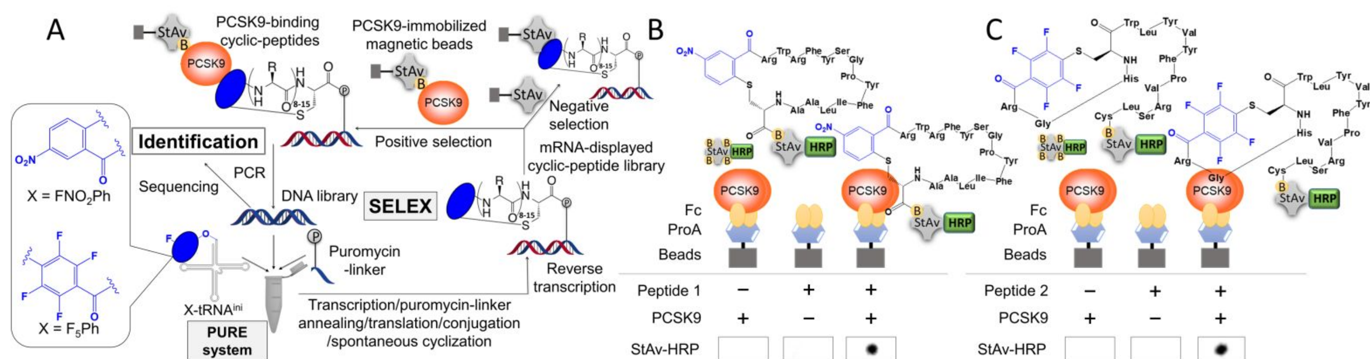

Fig. 2
(A) Schematic representation of SELEX of mRNA-displayed unnatural FNO_2Ph- and F_5Ph-cyclized peptides against PCSK9. (B) and (C) PCSK9-binding analysis of chemically synthesized FNO_2Ph- and F_5Ph-cyclized peptides 1 and 2.

PCSK9-binding activity than the original peptide dimer (Fig. S17).

PCSK9 on PVDF membranes used for immunoblotting could be also detected with FNO_2Ph-cyclized peptide dimers by HRP's chemiluminescence (Fig. S18). PCSK9 immobilized on beads could be fluorescently imaged using FNO_2Ph-cyclized peptide dimers labeled with Qdot 605 via streptavidin (Fig. S19). Finally, we applied FNO_2Ph-cyclized peptide dimers to sandwich ELISA of PCSK9 with anti-PCSK9 antibody. Free PCSK9 was captured with immobilized anti-PCSK9 antibody, and analyzed using PCSK9-binding FNO_2Ph-cyclized peptide dimers by HRP's chemiluminescence. Chemiluminescent analysis demonstrated that PCSK9 could be detected by sandwich ELISA with FNO_2Ph-cyclized peptide dimers and anti-PCSK9 antibody (Fig. S20).

In conclusion, we report two novel ribosomally incorporatable linkers for spontaneous peptide cyclization via nucleophilic aromatic substitution, FNO_2Ph and F_5Ph. These linkers showed more cysteine-specific cyclization than previously reported *N*-chloroacetyl amino acid and chloromethylbenzoic acid derivative linkers. We applied FNO_2Ph and F_5Ph to SELEX using PURE system-expressed, mRNA-displayed unnatural cyclic peptides, and identified two novel PCSK9-binding peptides. We also applied FNO_2Ph to *in vitro* affinity maturation. We synthesized chemical dimers of PCSK9-binding FNO_2Ph-cyclized peptides, and applied them to sandwich ELISA of PCSK9 with anti-PCSK9 antibody. Our results indicate that mRNA display SELEX of FNO_2Ph- and F_5Ph-cyclized peptides facilitates the discovery of novel artificial ligands for various therapeutic, diagnostic, and research applications. Nitro groups are usually undesired elements of drugs as this functional group is metabolically reduced to potentially cytotoxic groups. This issue can be addressed by substitution of the nitro group to different electron withdrawing group such as a sulfamoyl group. Further investigation in this context is currently under way in our laboratory.

This work was supported by JSPS KAKENHI (No. 15H05372), MEXT LEADER, ONO Medical Research Foundation, Sasakawa Scientific Research Grant, and Heiwa Nakajima Foundation, and in part by JST PRESTO (No. JPMJPR1685) and JSPS KAKENHI (No. 15KT0071 and 15K13751).

Conflicts of interest

T.K. have filed a provisional patent related to this work.

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