Ph.D. Thesis (in Engineering)

Construction of hybrid molecule libraries based on thioetherification of T7 phage-displayed peptides

“T7 ファージ提示ペプチドのチオエーテル化を基盤としたハイブリッド分子ライブラリーの作製”

Keisuke Fukunaga

March 2014

Department of Engineering Science,
The University of Electro-Communications (UEC),
Tokyo, Japan

Construction of hybrid molecule libraries based on thioetherification of T7 phage-displayed peptides

Judge members

- Dr. Haruki Niwa, professor at UEC
- Dr. Takayuki Ishida, professor at UEC
- Dr. Takashi Hirano, professor at UEC
- Dr. Hideki Shirakawa, associate professor at UEC
- Dr. Masumi Taki*, associate professor at UEC

* Chief examiner

Summary in Japanese

人工的な構造を持つペプチドライブラリーの作製が、近年、創薬分野において注目を集めている。特に、バクテリオファージに融合されたランダム化ペプチドに対して化学修飾を行うことで、ハイブリッド分子ライブラリーの作製が試みられている。しかしながら、既存の M13 ファージに対する化学修飾法では副反応や大腸菌感染能が低下するなどの問題が存在した。そこで、T7 ファージ提示ペプチドのチオエーテル化を試み、T7 ファージ提示ペプチド選択的なワンポット化学修飾を世界で初めて達成した。さらに、化学修飾した T7 ファージは大腸菌への感染能を損なわなかった。この技術を用いて異なる構造的特徴を持つハイブリッド分子ライブラリーを 3 種類構築し、任意の標的タンパク質に結合する分子のセレクション・機能解析を行った。

Preface

Since G.P. Smith reported the concept of phage display in 1985, the technique has been used as a powerful tool for construction of a peptide library. Currently, the phage display is applied for construction of a non-natural peptide library: peptide-artificial molecule hybrid library is constructed via post-translational chemical modifications of phage-displayed peptides.

Chapter 1 reviews features of two major phage display systems. In particular, sequence bias problems and practical tips on the library construction are discussed in detail.

The fist half of chapter 2 focuses on establishment of a novel method to construct the peptide-artificial molecule hybrid library. Here, I found that bacteriophage T7-displayed peptides are modifiable in a simplest method without side reaction and infectivity loss, whereas the site-specific modification of bacteriophage M13-displayed peptides are ultimately difficult.

From the latter half of the chapter 2 to chapter 4 focuses on construction of different kinds of the hybrid library and on screening of target-specific binders: In the chapter 2, fluorophore-conjugated hybrid molecule library is constructed, and glutathione *S*-transferase-specific binders are obtained. In the chapter 3, drug-like molecule-conjugated hybrid molecule library is constructed, and streptavidin-specific binders are obtained. In the chapter 4, crown ether-like macrocyclic library is constructed so that oligoethyleneglycol moiety has a good biocompatibility. Also, a novel binder against Hsp90, which is a drug target in cancer therapy, is obtained.

Abbreviations

5-TMRIA:	tetramethylrhodamine 5-iodoacetamide
$10BASE_d$-T:	gp10 based-thioetherification
Ac:	acetyl
BSA:	bovine serum albumin
CBB:	coomassie brilliant blue
CD:	circular dichroism
CID:	collision induced dissociation
CTD:	C-terminal domain
Da:	dalton
DNA:	deoxyribonucleic acid
DTT:	dithiothreitol
EBB:	*N,N'*-[1,2-ethanediyl-oxy-2,1-ethanediyl]bis(2-bromoacetamide)
ECL:	enhanced chemiluminescence
E. coli:	*Escherichia coli*
EDTA:	ethylenediaminetetraacetic acid
ELISA:	enzyme-linked immunosorbent assay
Em:	emission
ESI:	electrospray ionization
Ex.:	excitation
FITC:	fluorescein isothiocyanate
FL:	fluorescein
FP:	fluorescence polarization
FY:	financial year
GA:	geldanamycin
gp3:	gene-3 protein
gp10:	gene-10 protein
GST:	glutathione *S*-transferase
HEPES:	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC:	high performance liquid chromatography
HRP:	horseradish peroxidase
Hsp:	heat shock protein

IA:	iodoacetamide
IgG:	immunoglobulin G
ITC:	isothermal titration calorimetry
LB:	lysogency broth
LC:	liquid chromatography
Lys-C:	lysyl endopeptidase
NBD:	4-nitrobenzo-2-oxa-1, 3-diazole
NMR:	nuclear magnetic resonance
MD:	middle domain
MS:	mass spectrometry
NTD:	N-terminal domain
ODS:	octa decyl silyl
OEG:	oligoethyleneglycol
OPD:	*o*-phenylenediamine
PAGE:	poly acrylamide gel electrophoresis
PBS:	phosphate-buffered saline
PCR:	polymerase chain reaction
PEG:	polyethyleneglycol
PFU:	plaque-forming units
PVDF:	polyvinylidene difuoride
Sal:	salicylic acid
SDS:	sodium lauryl sulfate
TB:	terrific broth
TCEP:	Tris(2-carboxyethyl)phosphine
TMR:	tetramethylrhodamine
TOF:	time-of-flight
Tris:	tris(hydroxymethyl)aminomethane
UV:	ultraviolet

Table of contents

CHAPTER 1

Comparison of T7 and M13 systems in phage display

■ Abstract

Phage display technology is undoubtedly a powerful tool for affinity selection of target-specific peptide. Commercially available pre-made phage libraries allow us to take screening in the easiest way. On the other hand, construction of a custom phage library seems to be inaccessible, because several practical tips are absent in instructions. This chapter focuses on what should be born in mind for beginners using commercially available cloning kits (i.e., Ph.D. with type 3 vector and T7Select systems for M13 and T7 phage, respectively). In the M13 system, proline or basic amino acids (especially, Arg) should be avoided at the N-terminus of peptide fused to gp3. In both systems, peptides containing odd number(s) of cysteine(s) should be designed with caution. Also, DNA sequencing of a constructed library before biopanning is highly recommended for finding unexpected bias.

■ 1.1 Introduction

Phage display technology was born in 1985 when George Smith reported that foreign peptide could be displayed on the surface of filamentous bacteriophage.[1] Today, the phage display is a versatile tool for finding specific interactions between randomized library peptides/proteins on phage and target proteins, peptides, or other molecules. For example, it is applicable for generation of therapeutic peptides against cancer,[2] microbe,[3] novel functional protein,[4] or fully humanized monoclonal antibody.[5] The advantages of the phage display technology over other selection methods (*e.g.,* ribosome display) are: (i) cost of a routine is cheap. (ii) Time required for selection/amplification is fast. (iii) Extreme care for handling, such as RNA isolation/selection, is not necessary. The phage is a DNA-containing virus that infects bacteria, and makes many copies of the library within a very short time.[6]

A phage that specifically binds a target can be selected from mixtures of billions of phages, propagated by *in vivo* amplification, and then subjected to additional rounds of affinity selection (Fig. 1). This whole process is so called “biopanning”.[7] After multiple rounds of the biopanning, enrichment of target-binding phages can be assessed by phage titering and enzyme-linked immunosorbent assay (ELISA). Finally, the peptide displayed on the phage can be analyzed by DNA sequencing.

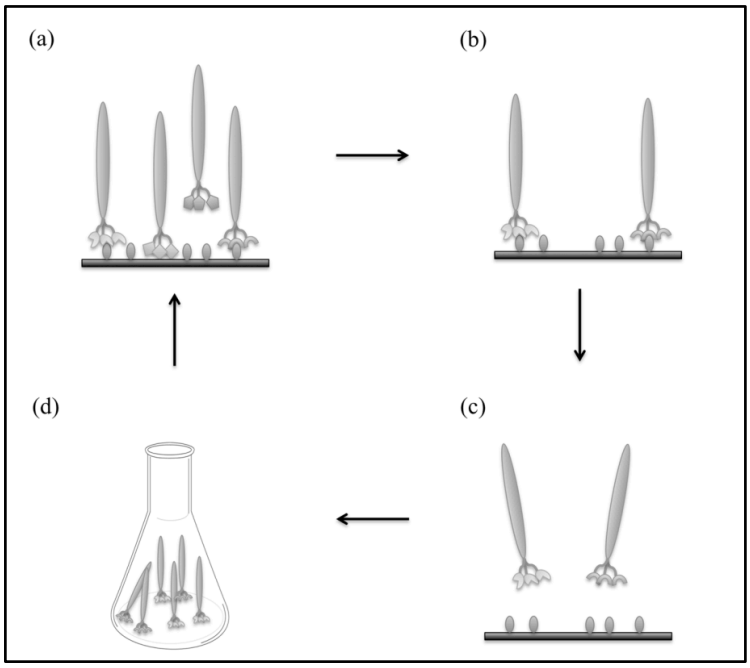


Figure 1. A typical procedure of the biopanning. (**A**) Incubation of phage library with an immobilized target. (**B**) Washing of unbound phage. (**C**) Elution of target-bound phage. (**D**) Amplification of the eluted phage for subsequent rounds of the biopanning.

Categorization of phage display systems.

Based on vector systems, the phage display systems can be categorized into two classes. One is a true phage vector system. The phage vector is often derived from genes encoding all phage proteins.[8] The library is to be cloned as a fusion with a component gene, which originally exists in the phage genome. Alternatively, some libraries are to be inserted in the same vector as an additional fusion gene encoding a displaying peptide and a phage protein.[9]

Another is a phagemid vector system. The phagemid is a plasmid containing both a phage-derived replication origin and a plasmid-derived one.[10] A phage containing the phagemid can be generated only when phage components are secreted from bacterial host carrying a helper phage. In this system, two types of phages could be theoretically produced carrying either phagemid genome or helper-phage one. Practically, a helper phage with defective replication origin is used for the generation of phage proteins; production of the helper phage itself will be suppressed. This system yields a phage

with the wild-type protein and library-fused one on the same virion, encoded by the helper phage and phagemid vector, respectively. Thus, numbers of the displaying peptides per virion from the phagemid system are less than those from the true vector system. This allows us to display not only small peptides but also large proteins,[11] which is beyond the scope of this chapter.

Among many different kinds of phages, M13 (filamentous bacteriophage) and T7 (lytic one) are exclusively used for the phage display. The M13 phage is composed of a circular single-stranded DNA genome and thousands copies of major capsid proteins (gp8), and capped by five copies of gp3 + gp6 on one end and five copies of gp7 + gp9 on the opposite (Fig. 2). The most widely-used M13 system is type 3. In this system, the peptide library is fused to the N-terminus of all five copies of the gp3. Other systems (*e.g.,* type 33, type 8 etc.) are categorized by a peptide-displaying protein on the M13 phage and numbers of peptides per virion (Table 1).[12, 13]

The T7 phage is an icosahedral-shaped phage with a capsid shell that is composed of 415 copies of gp10, linear double-stranded DNA, and other proteins (Fig. 2).[14] The gp10 includes two forms, gp10A (344 amino acids) and its frameshifting product, gp10B (397 amino acids).[15] In the T7 phage display systems, peptide library is always fused to the C-terminus of the gp10B. Numbers of peptides per virion and maximal size of the peptide are determined by the vector system (Table 1).[16]

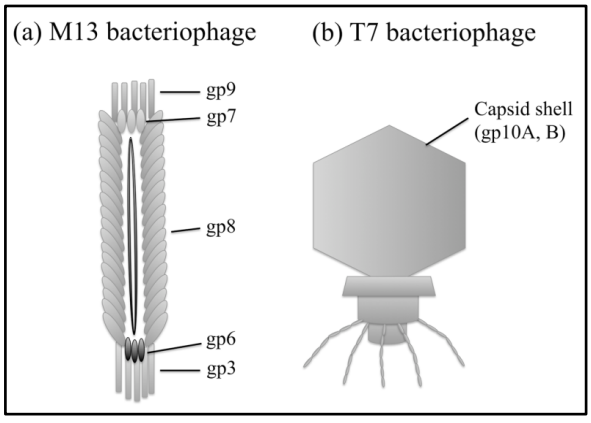


Figure 2. Structures of (**A**) the filamentous M13 bacteriophage, and (**B**) the lytic T7 bacteriophage.

Table 1. Features of various systems of M13 and T7 phages.

	System	Size limit	Numbers of peptides per virion	Presentation region
M13	3	unknown	5	N-terminus to gp3
	33	No limit	< 1	
	3 + 3			
	8	Short	> 2,700	N-terminus to gp8
	88	unknown	< 300	
	8 + 8	No limit	100-1000	
	8 + 8	unknown	< 1	C-terminus to gp8
	6 + 6	No limit		N-terminus to gp6
	6 + 6			C-terminus to gp6
	9 + 9			N-terminus to gp9
T7	T7Select1-1	1200 aa	< 1	C-terminus to gp10B
	T7Select1-2	900 aa		
	T7Select10-3	1200 aa	5 - 15	
	T7Select415-1	50 aa	415	

Table 2. Consignment services of phage display with in-house libraries.

Company name	Peptide design	Peptide structure
Creative Biolabs	X_{10}, X_{16}, or X_{20} [*1]	linear
Dyax	X_a-C-X_b-C-X_c [*1]	cyclic
Bicycle Therapeutics	X_a-C-X_b-C-X_c-C-X_d [*2]	cyclic with a non-natural linker

X stands for any randomized amino acid.

[*1] The library was built by varying 19 aa at the randomized positions; the codon encoding Cys is excluded.

[*2] Bicyclic peptide library was made via thioether linkages.[30]

Using pre-made phage libraries.

For screening, using a pre-made phage library is the most convenient way. Three types of M13 phage libraries, consisting of random linear/cyclic heptapeptides (Ph.D.-7 / Ph.D.-C7C) and linear dodecapeptides (Ph.D.-12), are commercially distributable from New England Biolabs Inc. (NEB). Several companies have constructed in-house pre-made peptide libraries; they provide screening services by using their phage libraries, instead of distributing ones. The chemical structures and features of the libraries are summarized in Table 2. Creative Biolabs Inc. even accepts a service contract from a commercial pre-made library (*e.g.,* Ph.D.-C7C system), a custom-constructed one in the company, or a hand-made one.

Construction of custom phage library.

Because of the limited kinds of resources, constructions of custom phage libraries are often performed by using kits available from NEB (Ph.D. Cloning System for M13 phage) or Merck Millipore (T7Select Cloning Kit for T7 one).[6] Although these instructions are well described, several practical tips are missing in both of them, which may lead beginners to pitfalls such as obtaining severe inherent bias of amino acid sequence in the randomized region. This chapter focuses on instant tips for the construction of peptide libraries and affinity selection by using the commercial resources.

■ 1.2 Characteristics of Ph.D. cloning system

Ph.D. cloning system is based on a type 3 vector of M13 phage encoding N-terminal library peptide fused to a minor coat protein, gp3.[17] Because gp3 plays a critical role for phage infection and randomized peptides are fused in all five copies of the gp3, infectivity of the M13 phage can be significantly affected by a sequence of the displaying peptide. Moreover, secretion of the M13 phage from *E. coli* closely depends on charges, hydrophilicity, and folding states of the displaying peptide.[18, 19] An amplification efficiency of the individual M13 phage clone is determined by a combination of the above infection and secretion rates. To avoid negative effects on the infection/secretion, one should be aware of the following in an insert DNA construction.

Signal peptidase cleavage.

Positively charged basic amino acids, lysine and arginine, near the signal peptidase cleavage site inhibit the secretion of phages[20] ; the cationic residue blocks translocation across the inner membrane of *E. coli*.[21] If the N-terminus of the displaying peptide should be positively charged, lysine has to be evidently chosen; 6 out of 99 arbitrarily-chosen clones of the commercial 12-mer library (Ph.D.-12) contained Lys at the terminus, whereas N-terminal Arg was never found in the same 99 clones.[22] If the N-terminal Arg is inevitable, using non-commercial prlA suppressor strains such as ARI180 or ARI182 may help to avoid the secY-dependent secretion problem.[20]

Pro at the terminus is also cumbersome. When proline is located next to the cleavage site, it inhibits the signal peptidase cleavage.[23, 24] Only one N-terminal Pro out of the 99 clones was found in the Ph.D.-12 library.[22]

If it is necessary to encode a specific amino acid sequence just after the signal peptidase cleavage site, prediction of the position-specific cleavage is recommended to avoid risks of inappropriate or insufficient cleavage. For example, an internet server, SignalP,[25] instantly does this, and I usually use 0.3 for the threshold D-cutoff value in the gram-negative bacteria mode.

If one does not have any favorites of particular N-terminal sequence just after the cleavage site, "R-E" or simple "A" should be the first choice. There is an overabundance of negatively charged amino acids (E and D) at +1 and +2, and A at +1, in gram-negative signal peptidase cleavage sites (Fig. 3).[22]

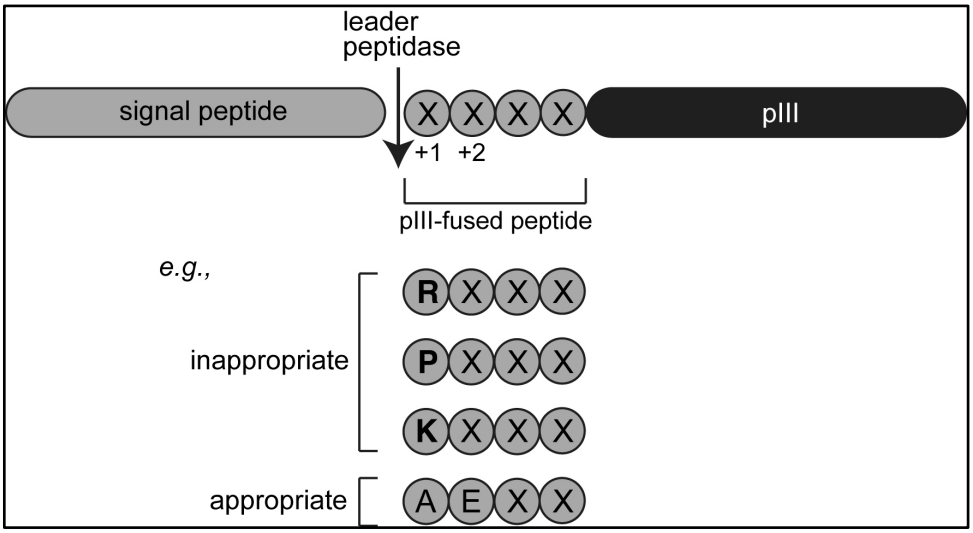


Figure 3. Sequence preference of the N-terminus of a peptide-pIII fusion (+1) in the M13 system. X stands for any randomized amino acid.

Unpaired Cys in a displaying peptide.

If one generates a custom phage library displaying a disulfide-constrained peptide, an insert DNA encoding even number(s) of Cys, but not odd number(s) should be designed. This is because an intramolecular disulfide (S-S) bond could be formed between an unpaired Cys in a displaying peptide and an intrinsic Cys in the gp3.[26] Phage assembly, infection, and/or secretion could be prevented by this unfavorable disulfide bond.[22, 27] It has been stated that an almost complete absence of odd number(s) of Cys was observed in the displaying peptide,[26, 28] which is also identical to my experience. For example, when I sequenced 10 independent M13 phage clones encoding Cys-X_7-Cys where the X stands for any randomized amino acid, no Cys was observed in the X_7 region; only the designated Cys at both ends seemed to form an intramolecular disulfide bond. Given the difficulty, if one still tries to generate a phage

library containing odd number(s) of Cys, M13 phages constructed by disulfide-free gp3[29, 30] might be useful without using the Ph.D. system.

■ 1.3 Characteristics of T7Select cloning system

Unlike the filamentous M13 system, T7 capsid shell displaying peptide library is not involved in phage infection, and/or secretion. Indeed, it has been proven that libraries of the T7 phages exhibit less sequence bias than those of the M13 ones.[27] This is a great advantage for library construction, because it is less necessary to pay attention to the amino acid sequences described above. The T7 system is also good at displaying a rigid motif with a hydrophobic domain, namely Trp cage.[31] This peptide motif was never displayed on the M13 system, presumably because the hydrophobic domain was anchored to the inner membrane of the *E. coli* prior to the phage assembly.[31]

Codon usage.

To the best of my knowledge, there is no description of a relationship between codon usage and bias against translation for the T7 system in *E. coli*; in the M13KE system, it is reported that rare codons of *E. coli* seldom affect the bias of peptide libraries.[22] To avoid potential risks that minor codons could stress the translation system,[32, 33] I simply use major codons (Table 3) for a non-randomized region of a synthetic DNA insert.

Table 3. Codon usage in *E. coli* K-12 strain.

Amino acid	Codon	CF (%)										
Phe	UUU	1.97	Ser	UCU	0.57	Tyr	UAU	1.68	Cys	UGU	0.59
	UUC	1.50		UCC	0.55		UAC	1.46		UGC	0.80
Leu	UUA	1.52		UCA	0.78	Stop	UAA	0.18	Stop	UGA	0.10
	UUG	1.19		UCG	0.80		UAG	0.00	Trp	UGG	1.07
	CUU	1.19	Pro	CCU	0.84	His	CAU	1.58	**Arg**	CGU	2.11
	CUC	1.05		CCC	0.64		CAC	1.31		CGC	2.60
	CUA	0.53		CCA	0.66	Gln	CAA	1.21		**CGA**	**0.43**
	CUG	4.69		CCG	2.67		CAG	2.77		**CGG**	**0.41**
Ile	AUU	3.05	Thr	ACU	0.80	Asn	AAU	2.19		**AGA**	**0.14**
	AUC	1.82		ACC	2.28		AAC	2.44		**AGG**	**0.16**
	AUA	**0.37**		ACA	0.64	Lys	AAA	3.32	Ser	AGU	0.72
Met	AUG	2.48		ACG	1.15		AAG	1.21		AGC	1.66
Val	GUU	1.68	Ala	GCU	1.07	Asp	GAU	3.79	Gly	GGU	2.13
	GUC	1.17		GCC	3.16		GAC	2.05		GGC	3.34
	GUA	1.15		GCA	2.11	Glu	GAA	4.37		GGA	0.92
	GUG	2.64		GCG	3.85		GAG	1.84		GGG	0.86

Codon frequency, CF. CF is defined as the percent frequency of each codon which matches in whole open-reading frame of the *E. coli* K-12 genome. Minor codons (bold letters; < 0.5%) could be avoided for insert DNA construction. This table was cited from codon usage database (http://www.kazusa.or.jp/codon/) with some modifications.

Unpaired Cys in a displaying peptide.

In my experiment, when a T7Select415-1b vector was used for the T7 packaging, the T7 phage failed to display a designated unpaired Cys. In this case, the library insert DNA was constructed using the genetic code of $(NNK)_6$-TGC-$(NNK)_6$, which encodes X_6-Cys-X_6. DNA sequencing of 8 independent phage clones revealed that peptides were

truncated by the appearance of a TAG stop codon before the designated Cys that was supposed to be translated (Fig. 4A).

The capsid shell used for randomized peptide display is composed of 415 copies of gp10.[34] A structural study of T7 procapsid shell suggested that the gp10 might play an important role in the interaction between capsid shell and scaffolding proteins.[35] The designated Cys in the library peptide fused to the gp10 might form an intermolecular disulfide bond with the same kind of unpaired Cys in a neighboring library peptide. It also might form an intramolecular one with an intrinsic Cys in the gp10. Too many unpaired Cys may inhibit proper/efficient assembly of the capsid shell proteins. Although I do not have direct evidence for this hypothesis, Rosenberg *et al.* also speculated that some peptide sequences might be unfavorable for the T7Select415 system.[16]

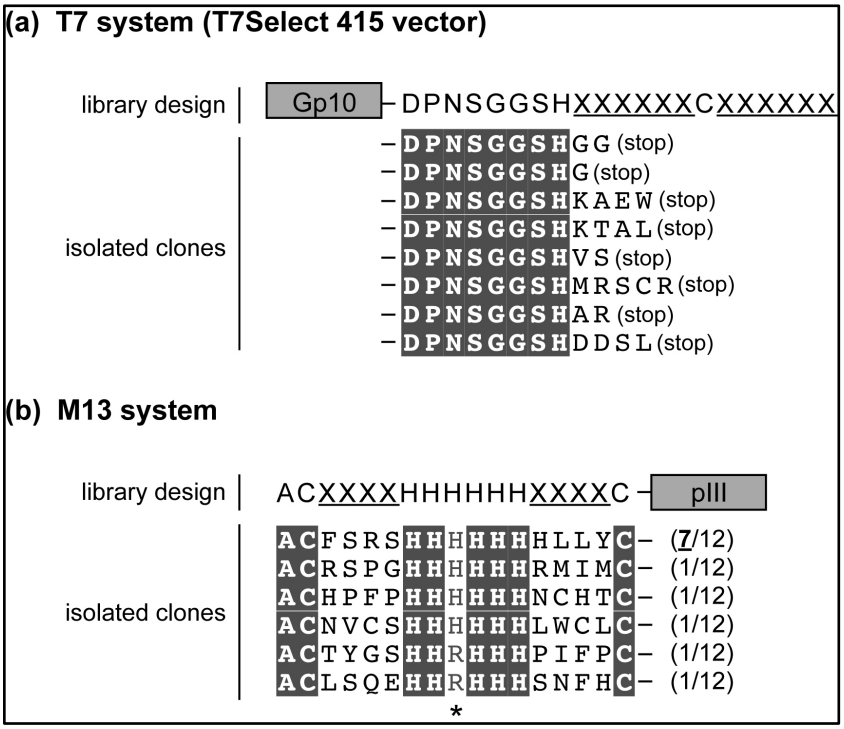


Figure 4. Unexpectedly isolated highly biased clones after library constructions. Underlined X indicate any amino acids. (**A**) Stop codon appearance before the designated Cys in the T7 system. A combination of T7Select415 vector and *E. coli* BL21 strain was used for *in vitro* packaging, and individual clones were subjected to DNA sequencing. For the sequence

alignment, ESPript program (http://espript.ibcp.fr/) was used. (**B**) Enrichment of a biased sequence and a mutation of the designated His. Asterisk indicates the mutated region. Randomly selected 12 individual M13 clones were subjected to DNA sequencing. Parentheses indicate numbers of the obtained clones.

Paired Cys in a displaying peptide.

Phages displaying the cyclic peptide by an intramolecular disulfide bond tend to exhibit higher target-binding ability, because their rigid structures minimize conformational entropy loss associated with the binding.[36, 37] Therefore, this kind of phage library is dominantly used for screening on the basis of not only M13 systems (*e.g.*, Ph.D. -C7C library from NEB) [36, 38] but also T7 ones.[39, 40] Disulfide constrained library of the T7 phage is most frequently constructed by using T7Select10-3b[27, 41] or 415-1b vector[42-45] (Table 2).

For generation of the disulfide constrained (S-S) library using the T7Select415 system, it is recommended in the manual (Merck Millipore) to use *E. coli* Origami B or Rosetta-gami B strains, which tends to enhance disulfide bond formation in the cytoplasm. However, these strains may not be required for the library constructions. By using *E. coli* BLT5615 strain included in the T7 kit with the T7Select10-3b[27] or 415-1b[45] vectors, the constrained library peptides were successfully displayed on the T7 phage, and high-affinity cyclic peptides were obtained.

Features of the T7 system.

One of the features of the T7 phage, which grows much faster than the M13 one, is that it decreases the time for phage titering and amplification. After infection, clear plaques of T7 phages will usually appear within 2-3 hours on LB plate with no additives. Liquid amplification of the T7 phage after affinity selection can also be conducted within the same time.

It is also attractive for beginners that the T7 system does not require any special instruments like an electroporator for the library construction. Contrary to the kit instructions, ultracentrifugation of the T7 phage with CsCl is not necessary for all

purification processes of ELISA assay and DNA sequencing. General procedure using polyethylene glycol (PEG)/NaCl with a conventional rotator is enough for the T7 phage purification, in the same way as the M13 system.

The T7 system can be useful for direct recovery of the highest affinity phage with a very slow off-rate from a target-linked solid support. It has been reported that a target-bound lambda phage can be directly amplified by the addition of *E. coli* in mid-log phase.[46] In a similar way, a library peptide displayed on the capsid shell does not interfere with the infectivity of the T7 phage. Indeed, I have experienced that a streptavidin-binding peptide containing the consensus sequence (H-P-Q)[47] was successfully obtained by this direct method (data not shown). In the M13 system, phages may also be eluted by the addition of the host bacterial cells, however the elution of the highest affinity binders may be hindered.

A minor drawback of the T7 system is that it is relatively expensive to construct a library with a high diversity. In a typical case, six whole tubes of T7 packaging extracts in a T7Select packaging kit (*ca.,* $410) are required to obtain a diversity of 4.1×10^8 PFU.[45]

Handling precautions.

It should be emphasized that *in vitro* packaging has to be performed with extreme care. One must keep a stringent condition of the temperature and mixing. Only “fresh” T7 packaging extract will make a high quality library; freezing and thawing of the extract will result in apparent reduction of the packaging efficiency.

Diluted T7 phages with a buffer or water tend not to be infective. It should be diluted with a buffer containing a protectant such as gelatin, or a growth media such as TB or LB.

■ 1.4 Importance of DNA sequencing for finding unfavorable bias and false-positives at an early stage of the affinity selection

After the electroporation for the Ph.D. system or the packaging for the T7Select system, a qualitative assessment of the phage library should be performed by DNA sequencing prior to the biopanning. I always confirm it by a conventional DNA sequencer with at least 10 independent phage clones. For example, I obtained highly biased sequences when the random library encodes constraints with a His_6-tag (A-C-X_4-H_6-X_4-C) (Fig. 4B). In this case, a specific sequence was predominantly enriched (C-F-S-R-S-H-H-H-H-H-H-H-L-L-Y-C; 7 out of 12 arbitrarily-chosen clones). In addition, one of the designated His at the 3rd position of the His_6-tag was mutated to Arg accompanied with a codon replacement from CAC to CAT. Nature seems to exclude the constrained His-tag in the M13 system, and such a library should not be used for the biopanning.

Advantage of high-throughput DNA sequencing.

A next generation sequencer (NGS) makes it possible to sequence millions of inserts in parallel. If the NGS is available, one million reads of the library clones would be ideal for finding target-binding sequences even after first round of the biopanning (Fig. 5).[48] If false positive sequences such as target-unrelated (*e.g.*, plastic or BSA) binders or propagation accelerating peptide (*e.g.*, H-A-I-Y-P-R-H)[49] are predominantly enriched at an early stage, further biopanning will be useless. These meaningless false-positive sequences are well-described and summarized in a recent-published review,[50] and can be found easily with online databases (SAROTUP[51], http://immunet.cn/sarotup/; PepBank[52], http://pepbank.mgh.harvard.edu/). Once candidate clones are selected after several rounds of biopanning, the false positive sequences should be excluded in the same manner. If the DNA sequencing is performed by a conventional sequencer but not by the NGS, one should be aware that the DNA sequencing of 50 randomly-chosen clones after first or second rounds of the biopanning would be completely

uninformative for finding target binders, because the population will be lacking[48]; it should be performed at a later round.

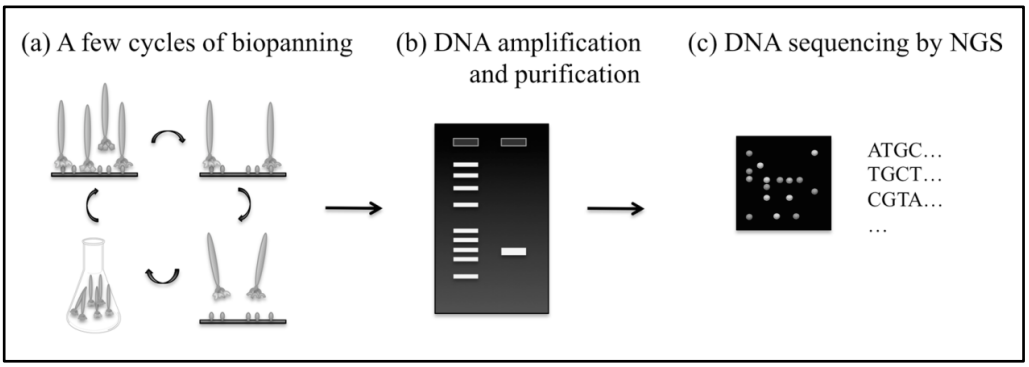


Figure 5. Phage display screening with next generation sequencing. (**A**) Biopanning with one or two-cycle(s). (**B**) Randomized-region of phage DNA is amplified with Polymerase Chain Reaction (PCR). The products are subjected to gel electrophoresis followed by further DNA purification. (**C**) Purified DNA is analyzed by a next generation sequencer.

■ 1.5 Conclusions

Merits and demerits of the M13 and T7 systems are summarized in Table 4. It seems that the T7 system is easier to handle for beginners, because there are several engineering tolerances. Additionally, the T7 phage is stable to detergents and denaturants (*e.g.*, 1% sodium dodecyl sulfate (SDS), urea (up to 4 M), and guanidine-HCl (up to 2 M)), for eliminating non-specific binders during the biopanning. Although the T7 phage is robust against not only the chemicals but also an alkaline condition (pH 10), it is fragile at acidic conditions below pH 4. If an elution from target-linked solid support under the lower pH is necessary, the M13 system should be the first choice.

In both systems, the DNA sequencing of a constructed phage library before biopanning is highly recommended for finding unexpected bias.

Table 4. Comparison of the M13 and T7 phage in library construction and affinity selection.

	M13 phage	T7 Phage
Cost	routinely cheap, requires electroporator and cuvettes	routinely expensive, no additional instruments required
Site of library peptide	N-terminus of gp3	C-terminus of gp10B
Library size / mg DNA	~10^9	~10^8 [45]
Peptide sequence bias	highly biased	less biased
Time required for phage titering/amplification	long	short

■ 1.6 References

[1] G.P. Smith, *Science*, 1985, **228**, 1315-1317.

[2] R. Brissette, J.K. Prendergast, N.I. Goldstein, *Curr. Opin. Drug Discov. Develop.*, 2006, **9**, 363-369.

[3] T.K. Lu, M.S. Koeris, *Curr. Opin. Microbiol.,* 2011, **14**, 524-531.

[4] A. Skerra, *FEBS J.*, 2008, **275**, 2677-2683.

[5] N. Lonberg, *Curr. Opin. Immunol.*, 2007, **20**, 450-459.

[6] L.R. Krumpe, T. Mori, *Int. J. Pep. Res. Therap.*, 2006, **12**, 79-91.

[7] E. Koivunen, W. Arap, D. Rajotte, J. Lahdenranta, R. Pasqualini, *J. Nucl. Med*., 1999, **40**, 883-888.

[8] M. Russel, *Trends Microbiol.*, 1995, **3**, 223-228.

[9] T. Haaparanta, W.D. Huse, *Mol. Diversity*, 1995, **1**, 39-52.

[10] T. Bratkovic, *Cell. Mol. Life Sci.*, 2010, **67**, 749-767.

[11] M. Russel, H.B. Lowman, T. Clackson, *Phage display -A practical approach*, Oxford University Press, 2004, **266**, 1-26.

[12] G.P. Smith, V.A. Petrenko, *Chem. Rev.*, 1997, **97**, 391-410.

[13] V.A. Petrenko, G.P Smith, *Phage Display in Biotechnol. Drug Discov.*, 2005, 63-98.

[14] X. Agirrezabala, J. Martin-Benito, J.R. Caston, R. Miranda, J.M. Valpuesta, J.L. Carrascosa, *EMBO J*., 2005, **24**, 3820-3829.

[15] B.G. Condron, J.F. Atkins, R.F. Gesteland, *J. Bacteriol.*, 1991, **173**, 6998-7003.

[16] A. Rosenberg, K. Griffin, F.W. Studier et al., *inNovations*, 1998, **6**, 1-6.

[17] K.A. Noren, C.J. Noren, *Methods*, 2001, **23**, 169-178.

[18] G.A. Barkocy-Gallagher, J.G. Cannon, P.J. Bassford, Jr., *J. Biol. Chem.*, 1994, **269**, 13609-13613.

[19] E.H. Manting, A.J. Driessen, *Mol. Microbiol.*, 2000, **37**, 226-238.

[20] E.A. Peters, P.J. Schatz, S.S. Johnson, W.J. DoIr, *J. Bacteriol.*, 1994, **176**, 4296-4305.

[21] H. Andersson, G. von Heijne, *Proc. Natl. Acad. Sci. USA*, 1991, **88**, 9751-9754.

[22] D.J. Rodi, A.S. Soares, L. Makowski, *J. Mol. Biol.,* 2002, **322**, 1039-1052.

[23] I. Nilsson, G. von Heijne, *FEBS Lett.*, 1992, **299**, 243-246.

[24] A. Pluckthun, J.R. Knowles, *J. Biol. Chem.*, 1987, **262**, 3951-3957.

[25] T.N. Petersen, S. Brunak, G. von Heijne, H. Nielsen, *Nat. Methods*, 2011, **8**, 785-786.

[26] S.J. McConnell, A.J. Uveges, D.M. Fowlkes, D.G. Spinella, *Mol. Div.*, 1996, **1**, 165-176.

[27] L.R. Krumpe, A.J. Atkinson, G.W. Smythers et al., *Proteomics*, 2006, **6**, 4210-4222.

[28] B.K. Kay, N.B. Adey, Y.S. He, J.P. Manfredi, A.H. Mataragnon, D.M. Fowlkes, *Gene*, 1993, **128**, 59-65.

[29] I. Kather, C.A. Bippes, F.X. Schmid, *J. Mol. Biol.*, 2005, **354** (3), 666-678.

[30] C. Heinis, T. Rutherford, S. Freund, G. Winter, *Nat. Chem. Biol.*, 2009, **5**, 502-507.

[31] R.E. Herman, D. Badders, M. Fuller et al., *J. Biol. Chem.*, 2007, 282, 9813-9824.

[32] X. Wu, H. Jornvall, K.D. Berndt, U. Oppermann, *Biochem. Biophys. Res. Commun.*, 2004, 313, 89-96.

[33] A. Hilterbrand, J. Saelens, C. Putonti, *BMC Bioinform.*, 2012, 13, 62.

[34] J.J. Steven, B.L. Trus, *Electr. Microscop. Prot.*, 1991, **5**, 1-35.

[35] X. Agirrezabala, J.A. Velazquez-Muriel, P. Gomez-Puertas, S.H. Scheres, J.M. Carazo, J.L. Carrascosa, *Structure*, 2007, **15**, 461-472.

[36] M.A. McLafferty, R.B. Kent, R.C. Ladner, W. Markland, *Gene*, 1993, **128**, 29-36.

[37] R.C. Ladner, *Trends Biotechnol.*, 1995, **13**, 426-430.

[38] K. Tanaka, M. Nishimura, Y. Yamaguchi et al., *J. Neuroimmunol.*, 2011, **236**, 27-38.

[39] H.B. Lowman, *Ann. Rev. Biophys. Biomol. Struct.*, 1997, **26**, 401-424.

[40] F. Uchiyama, Y. Tanaka, Y. Minari, N. Tokui, *J. Biosci. Bioeng.*, 2005, **99**, 448-456.

[41] K. Sakamoto, Y. Ito, T. Hatanaka, P.B. Soni, T. Mori, K. Sugimura, *J. Biol. Chem.*, 2009, **284**, 9986-9993.

[42] P. Laakkonen, K. Porkka, J.A. Hoffman, E. Ruoslahti, *Nat. Med.*, 2002, **8**, 751-755.

[43] M. Essler, E. Ruoslahti, *Proc. Natl. Acad. Sci. USA*, 2002, **99**, 2252-2257.

[44] H. Witt, K. Hajdin, K. Iljin et al., *Int. J. Cancer*, 2009, **124**, 2026-2032.

[45] X. Fan, R. Venegas, R. Fey et al., *Pharm. Res.*, 2007, **24**, 868-879.

[46] Y.G. Mikawa, I.N. Maruyama, S. Brenner, *J. Mol. Biol.*, 1996, **262**, 21-30.

[47] B.A. Katz, *Biomol. Engin.*, 1999, **16**, 57-65.

[48] P.A. 't Hoen, S.M. Jirka, B.R. Ten Broeke et al., *Anal. Biochem.*, 2012, **421**, 622-631.

[49] L.A. Brammer, B. Bolduc, J.L. Kass, K.M. Felice, C.J. Noren, M.F. Hall, *Anal. Biochem.*, 2008, **373**, 88-98.

[50] M. Vodnik, U. Zager, B. Strukelj, M. Lunder, *Molecules*, 2011, **16**, 790-817.

[51] J. Huang, B. Ru, S. Li, H. Lin, F.B. Guo, *J. Biomed. Biotechnol.*, 2010, **2010**, 101932.

[52] T. Duchrow, T. Shtatland, D. Guettler, M. Pivovarov, S. Kramer, R. Iissleder, *BMC Bioinform.*, 2009, **10**, 317.

CHAPTER 2

Construction of a tetramethylrhodamine-conjugated peptide library and selection of glutathione *S*-transferase-binders

■ Abstract

Phage display technology is a powerful tool to construct peptide library and to screen a target-specific binder. However, the displayed peptide cannot always have an optimal structure to interact with a target of interest. Post-translational chemical modification is an approach to get over this problem. By using the method, one peptide library can be perfectly converted to another library, which may contain the optimal molecule for the interaction. In this chapter, site-specific conjugation with a haloacetamide derivative into a designated cysteine on a displaying peptide on a capsid protein (gp10) of bacteriophage T7 has been achieved. This easiest gp10-based thioetherification (10BASE$_d$-T) is carried out in one-pot without side reactions or loss of phage infectivity. Here, tetramethylrhodamine-conjugated library is constructed, and glutathione *S*-transferase-specific binders are obtained from the library.

■ 2.1 Introduction

Recently, construction of a peptide library containing non-natural structures has been attracting much attention for drug discovery. Most frequently, non-natural amino acids as artificial structures are incorporated into specific position(s) of the peptide by an *in vitro* translation system along with genetic code reprogramming.[1-4] This sophisticated system has a great advantage in terms of peptide diversity. However, usage of an expensive cell-free translation system as well as fragile RNAs is required for the library construction. Another problem is that not all non-natural amino acids can be incorporated because *E. coli* ribosome excludes certain structures of the amino acids such as large aromatic ones.[6]

Post-translational chemical modification of a phage-displayed peptide is an alternative approach for the construction of a non-natural peptide library.[7, 8] Although the diversity of the phage library is smaller than the *in vitro* translated one, the phage display technology[10] is more accessible in terms of cost and handling. A pioneering work of the non-natural peptide library construction on the phage is reported by Winter's group in 2004. They successfully developed a fluorogenic biosensor by conjugating a fluorophore with a designated Cys at the antigen-binding site of a phage-displayed antibody library.[11] To date, Heinis has preceded this field with chemically constrained bicyclic peptides on the phage.[12-14] In such cutting-edge studies, M13 phage has been exclusively used until today. However, the library contains an inherent bias of amino acid sequences because secretion of M13 phage from *E. coli* closely depends on charges, hydrophilicity, and folding states of the M13-displayed peptides.[15-17] Moreover, it is reported that the side reaction against the intrinsic Cys of the minor coat protein (gp3) occurred in the post-translational chemical modification.[12] In my independent study, not only gp3 but also various M13 phage proteins were modified (Fig. 1), suggesting that site-specific modification of the M13-displayed peptide was extremely difficult. To avoid the side reaction, genetically engineered M13 phage carrying Cys-free gp3 is often used.[12-14, 18] Even though the site-specific chemical

modification of the M13-displayed peptides is achieved, it results in a significant decrease of the infectivity titer.[12]

The T7 system is also used for the phage display,[19] and is superior to the M13 one in view of the sequence bias and growth rate.[17, 20] A recent structural study of the T7 major capsid protein (gp10) reveals that two intrinsic Cys of the gp10 are buried inside of the mature capsid (Fig. 2).[21] On the other hand, the library peptide fused to the C-terminus of the gp10 has to be located outside of the capsid to come in contact with various targets. These facts motivated us to carry out straightforward chemical modification of the T7 phage-displayed peptide without construction of Cys-free gp10; because of steric hindrance, the intrinsic Cys will not be modified. Here the introduction of non-natural groups into a designated Cys on the T7-displayed peptide, namely gp10 based-thioetherification (10BASE$_d$-T), was performed as shown in Schemes 1 and 2.

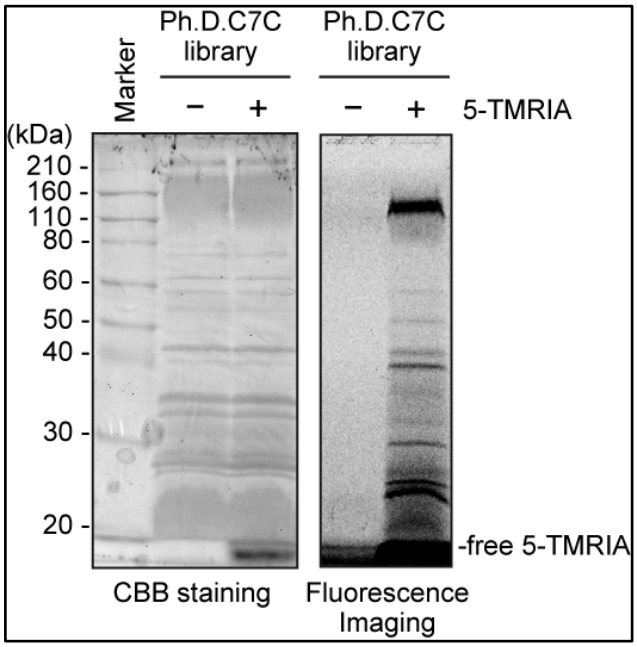


Figure 1. Chemical modification against M13-displayed peptide library. 5-TMRIA was used for the modification. Experimental condition was the same as 10BASE$_d$-T. Ph.D. -C7C phage display peptide library was purchased from New England Biolabs (cat. No. E8120S).

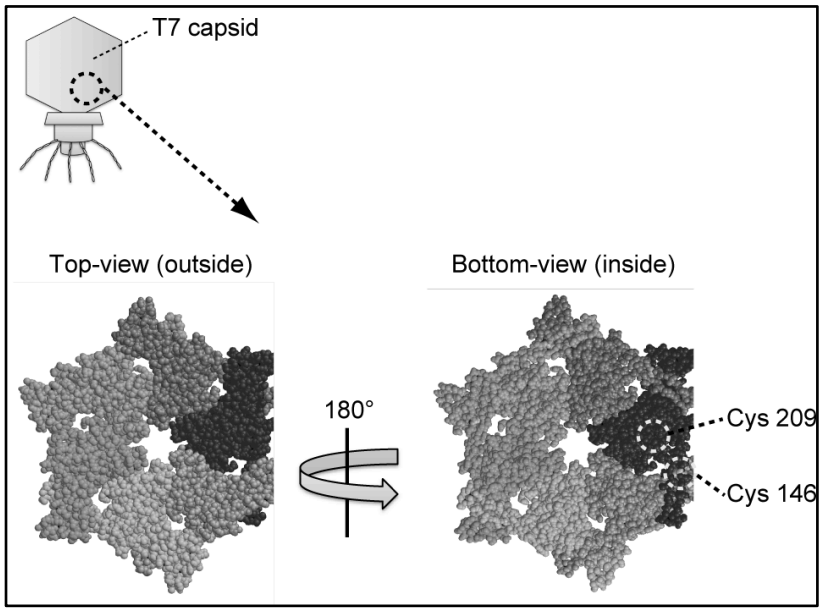
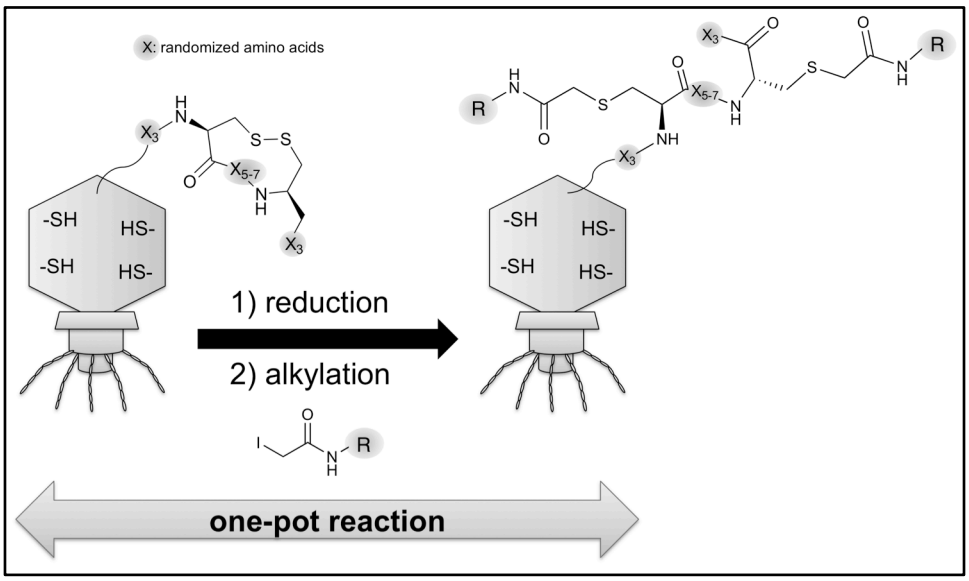
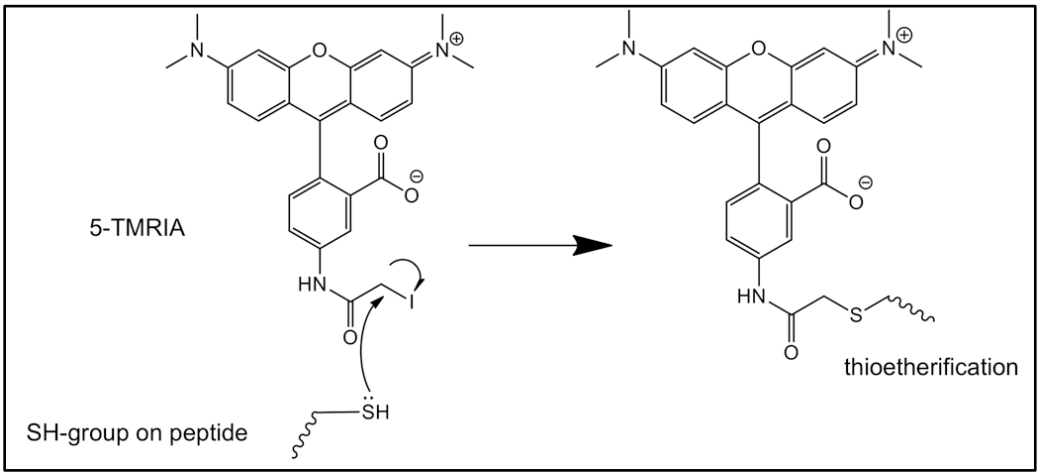


Figure 2. A structural model of hexametric gp10 (RCSB Protein Data Bank ID: 2XVR) revealed that the intrinsic thiol groups of the gp10 (Cys146 and Cys209) are buried inside of the mature capsid.[1] The structures were generated by RasMol software (http://rasmol.org/).



Scheme 1. Site-specific introduction of a non-natural group (R) into the designated Cys on a T7 phage-displayed peptide by the 10BASE$_d$-T.



Scheme 2. Thioetherification of SH-group on peptide by S_N2 reaction.

■ 2.2 Results and discussion

Tetramethylrhodamine-5-iodoacetamide (5-TMRIA) was used as a model alkylating reagent, because it can be easily analyzed by fluorescence imaging, liquid chromatography (LC), and fluorescence polarization (FP) assay. For the site-specific introduction of tetramethylrhodamine (TMR) via the $10BASE_d$-T, I only mixed 5-TMRIA (200 μM) with the T7-displayed peptide (1.0×10^{11} plaque forming units of the T7 phage) in 700 μL of phosphate-buffered saline (pH 7.4) supplemented with 500 μM tris(2-carboxyethyl)phosphine (TCEP) and 400 mM NaCl at 4°C. After 3 hours of reaction, whole T7 phage proteins were subjected to sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE), followed by fluorescence imaging. A single fluorescent band could be seen at an appropriate molecular weight (*ca.,* 44 kDa) of the peptide-fused gp10 (Fig. 3A; upper panel, lane 3). This indicates that the alkylation exclusively occurred at the peptide-fused gp10. Note that neither protein components required for the infection nor other T7 phage molecules were reacted. Several reports stated that trialkylphosphines unfavorably react with haloacetic acids and their amide derivatives.[22, 23] Thus, the reduction and alkylation are usually performed in two separate steps.[11, 12, 24] Contrary to popular belief, chemical modification of the peptides with TCEP and 5-TMRIA could be performed in one-pot without tedious purification of the reduced intermediate. For site-specific modification of the T7-displayed peptides, the optimal molar concentration of 5-TMRIA was around 200 μM; higher concentration gave non-specific modification (Fig. 4). To quantitate the efficiency of the $10BASE_d$-T, Western blot analysis was performed by using a FLAG epitope, which is located between gp10 and the T7-displayed peptide. After the modification, the original band of the FLAG-tagged protein almost disappeared. Instead, a band of a slightly higher molecular weight appeared (Fig. 3A; lower panel, lanes 1 and 3). In the Western blotting-based quantification, it was estimated that at least 95% of the peptide-fused gp10 had been modified (data not shown). Next, I examined whether TMR was conjugated only to the T7-displayed peptides but not to the intrinsic

Cys of gp10. To confirm this, the modified peptide-fused gp10 was site-specifically cleaved at the end of the FLAG-tag by enterokinase. As expected, the fluorescence band totally disappeared after enterokinase digestion (Fig. 3A; upper panel, lanes 3 and 4), suggesting that TMR was appropriately conjugated only to the T7-displayed peptide.

To achieve more precise characterization, I performed a mass spectrometric analysis of the fluorophore-conjugated peptide. MS analysis of the phage-displayed peptide is challenging because a small amount of peptides is obtained in a batch.[8] In the case of the T7 phage, only *ca*. 10 molecules of the peptides are displayed per single virion, when a mid-copy phage display vector (T7Select10) is used.[25] To bypass this problem, I generated a model T7 phage carrying approximately 200 molecules of the peptides per single virion[26] by using a high-copy vector (T7Select415). After the 10BASE$_d$-T followed by SDS-PAGE, the fluorescent band in the gel was excised (Fig. 3B) and digested with trypsin. The resulting peptide fragments were analyzed by LC-MS/MS. Two chromatographic peaks at 550 nm (TMR absorption) were detected (Fig. 3C; upper panel), and each of them was identified as the TMR-conjugated T7-displayed peptide by tandem mass spectrometry (Fig. 3C; lower panel). Also these data suggest that the two TMR molecules were conjugated to the two designated Cys on a single T7-displayed peptide. The TMR-conjugation was also validated by LC-MS analysis of the lysyl endopeptidase-digested sample (Fig. 5). At the same time, most of the other peptide fragments derived from gp10 were identified by peptide mass fingerprinting based on MS/MS ion search (data not shown).

Next, I performed the 10BASE$_d$-T on a T7 phage library generated from the mid-copy vector. In-gel fluorescence imaging and Western blot analysis should that almost all of the peptide-fused gp10 were modified (Fig. 3D). On the other hand, a small amount of the peptide-fused gp10 remained at the same original position (Fig. 3D; lower panel). These faint bands were considered to be truncated peptide-fused gp10 without Cys as shown in Fig. 6 (lanes 2 and 6). These data indicate that the

T7-displayed peptides can be modified in a sequence independent manner only if Cys is present in the peptide.

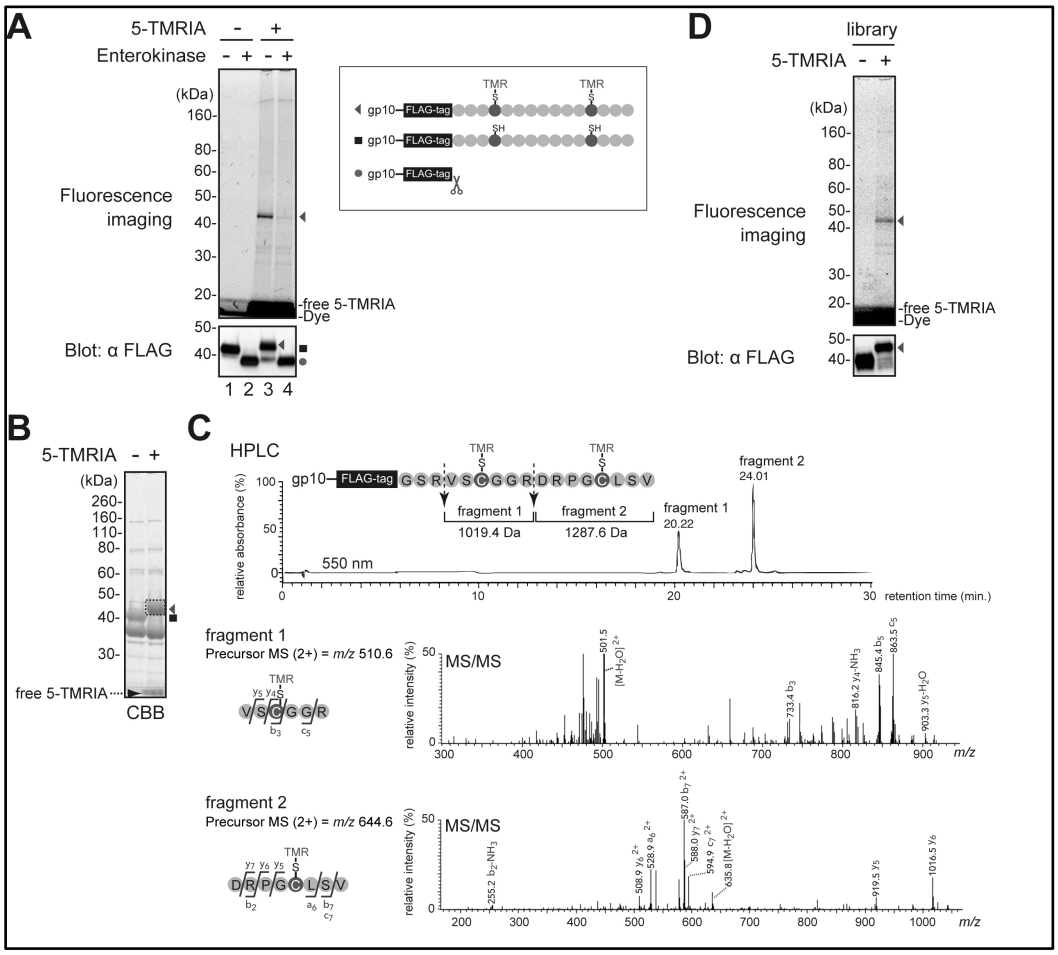


Figure 3. (**A**) Fluorescence imaging of TMR-modified T7 phage proteins, and site-specific cleavage of peptide-fused gp10 by enterokinase. Here, a model T7 phage carrying a mid-copy vector was modified via the 10BASE$_d$-T. Western blotting against FLAG-tag (lower panel) was performed with the gel shown in above panel. Triangles, squares, and circles represent TMR-conjugated peptide-fused gp10, intact one, and enterokinase-digested one, respectively. (**B**) Introduction of TMR onto T7-displayed peptide via the 10BASE$_d$-T. Here, a model T7 phage carrying a high-copy vector was used. The dashed square corresponding to TMR-conjugated peptide-fused gp10 was excised and subjected to in-gel trypsinization followed by LC-MS/MS analysis. CBB indicates coomassie brilliant blue staining. (**C**) Identification of peptide fragments derived from the TMR-conjugated T7-displayed peptide by LC-MS/MS. Upper panel: 550 nm (TMR absorption) chromatogram of the trypsinized peptide-fused gp10. Middle and lower panel: MS/MS spectrum corresponding to each of the two chromatographic peaks. Note that trypsin

could not cleavage before proline.[9] (**D**) TMR-modification of T7-displayed library peptides via the 10BASE$_d$-T.

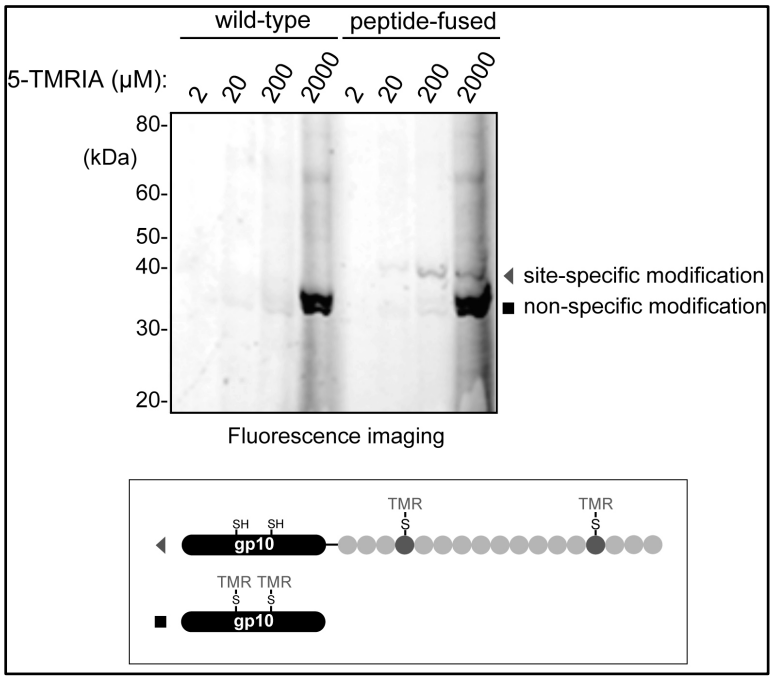


Figure 4. Optimization of 5-TMRIA concentrations. The T7 phage clones were modified via the 10BASE$_d$-T with various molar concentrations of 5-TMRIA under the standard condition (described in Materials and Methods). Equal amounts of phage proteins were subjected to SDS-PAGE followed by fluorescence imaging.

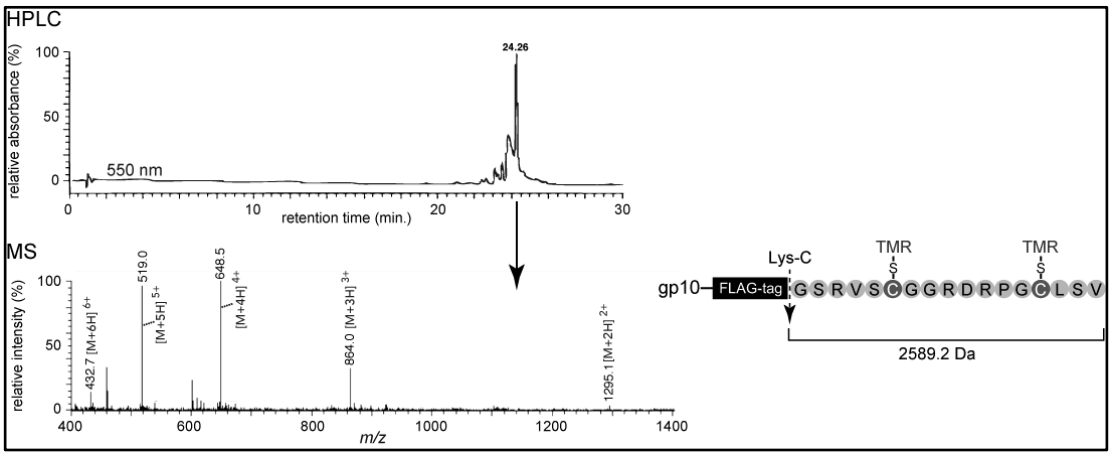


Figure 5. LC-MS analysis of lysyl endopeptidase-digested gp10 fusion. Upper panel: HPLC chromatogram of the digested gp10. The 550 nm absorption corresponding TMR absorption was

monitored. Lower panel: MS spectrum of the corresponding peak at 24.26 min. A series of multiple charged ions were detected (experimental *m/z* values 1295.1, 864.0, 648.5, 519.0, and 432.7), and consistent with theoretical *m/z* values of the TMR-conjugated peptide (calculated *m/z* values 1295.6, 864.1, 648.3, 518.8, and 432.5). Lys-C represents lysyl endopeptidase.

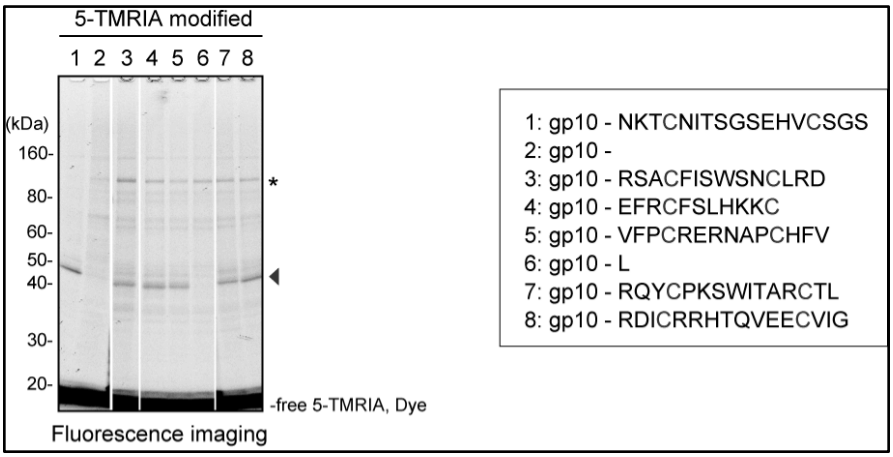


Figure 6. Introduction of tetramethylrhodamine (TMR) into different peptide-fused gp10. Amino acid sequences of the peptides are shown in right panel. A triangle and an asterisk (*) represent the TMR-conjugated gp10 and contaminated protein derived from *E. coli*, respectively. Two clones lacking Cys in T7 phage-displayed peptide were not modified (clones 2 and 6). In case of these clones, T7-displayed peptides were truncated by the appearance of a stop codon before the designated Cys that was supposed to be translated.

When using a phage-displayed peptide library along with the post-translational chemical modifications, one concern is the negative effect on infectivity for *E. coli*.[8] Therefore, I examined the infectivity of the TMR-conjugated T7 phage peptide library by plaque assay. As shown in Fig. 7, the modified T7 phage completely retained its infectivity. A similar observation has been recently reported; an enzyme treated-T7 phage fully retains the infectivity, whereas the treated-M13 phage does not. These data suggest that the T7 phage is an excellent platform for post-translational modifications.

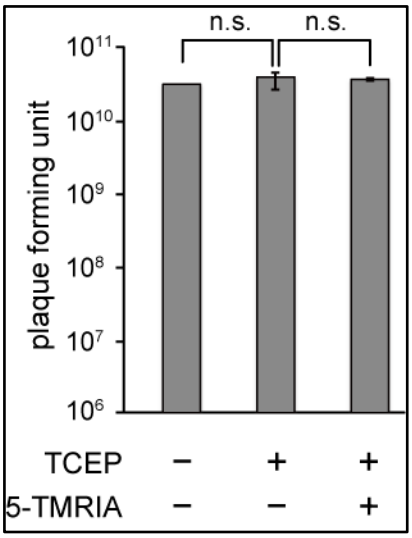


Figure 7. Infectivity of modified T7 phage. A T7 phage library was treated with or without 5-TMRIA in the presence of TCEP under the standard condition (described in Experimental selection). The number of plaque forming units was determined by serial dilution method and plaque assay. The graph summarizes the results of three independent experiments. Error bars represent standard deviations. Statistical analysis was performed by unpaired Student's *t*-test. n.s., not significant (*p* values = 0.17 [left] and 0.31 [right], respectively).

I next attempted to find target-protein specific binders from TMR-conjugated peptide libraries (Fig. 8). As a model target protein, I chose glutathione *S*-transferase (GST) because it is thought to be a potential therapeutic target for cancer.[28, 29] Prior to biopanning, mixed T7 libraries carrying X_3-C-X_{5-7}-C-X_3 peptides (where X represents any amino acid) were modified through the 10BASE$_d$-T. To include the possibility that a doubly-alkylated peptide may improve the affinity toward the target protein, I conjugated two TMRs into each library peptide with two Cys. Five rounds of biopanning were performed against biotinlylated-GST, and enrichment of the GST-binders was confirmed by enzyme-linked immunosorbent assay (ELISA). To examine whether the amide or the TMR moiety is required for the binding, 5-TMRIA-treated T7 phage polyclones and iodoacetamide (IA)-treated ones were subjected to ELISA in parallel. After 5 rounds, the TMR-conjugated T7 phage polyclones should the strongest binding to GST (Fig. 9A). This indicates that the TMR moiety of the peptides plays a crucial role in interaction with GST. I found that all 5

clones randomly chosen from the phage pool after 5 rounds had a consensus sequence of C*-X-X-D-G-Y/F (C* and X represent alkylated Cys and any amino acid, respectively) (Fig. 9B). Among them, 3 clones bound only to GST when it was modified with 5-TMRIA but not IA (Fig. 9B and 10; clones 1, 4, and 5). Thus, I chemically synthesized one of them, TMR-conjugated Ac-R-M-W-C*-T-H-D-G-Y-OH peptide (equivalent to clone 5 shown in Fig. 9B and 10), and examined its binding affinity to GST by FP assay. GST-specific binding of the TMR-conjugated peptide was observed (Fig. 9C), whereas negative control peptides did not bind to GST (Fig. 11). The dissociation constant (K_D) of the TMR-conjugated peptide was estimated to be 3.6 μM (Fig. 9C).

Glutathione (GSH) is a well-known GST-binding tripeptide. I next examined whether the TMR-conjugated peptide inhibits GSH-GST interaction. But there are two possible interfere mechanisms: one is competitive binding. Another is structure change of the TMR-conjugated peptide and/or GST under reducing condition, because GSH could behave as a reducing agent. To exclude the possibility that reducing condition interfere with association of the TMR-conjugated peptide with GST, TCEP was used as mock competitor. By addition of GSH but not TCEP, the interaction was abolished (Fig. 12A), indicating that the TMR-conjugated peptide would be a GSH competitor. This was confirmed by competitive elution of GST from GSH-coupled beads (Fig. 12A). However, I cannot exclude the possibility of allosteric inhibition.

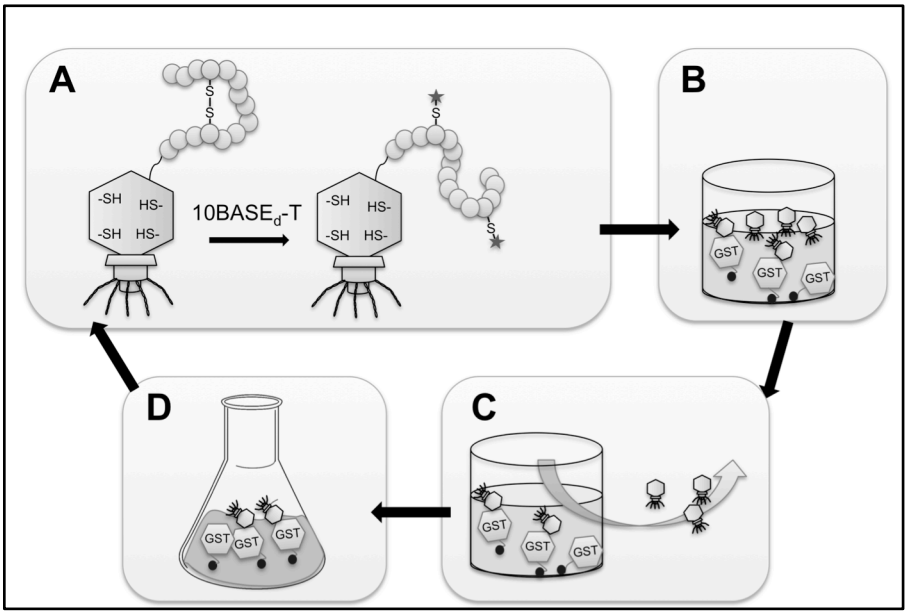


Figure 8. Schematic diagram of biopanning used in this study. (**A**) Construction of TMR-conjugated peptide library on T7 phage through the 10BASE$_d$-T. (**B**) Incubation of the phage display peptide library with immobilized glutathione *S*-transferase (GST). (**C**) Washing of unbound phage. (**D**) Amplification of the GST-bound phage for subsequent rounds of the biopanning.

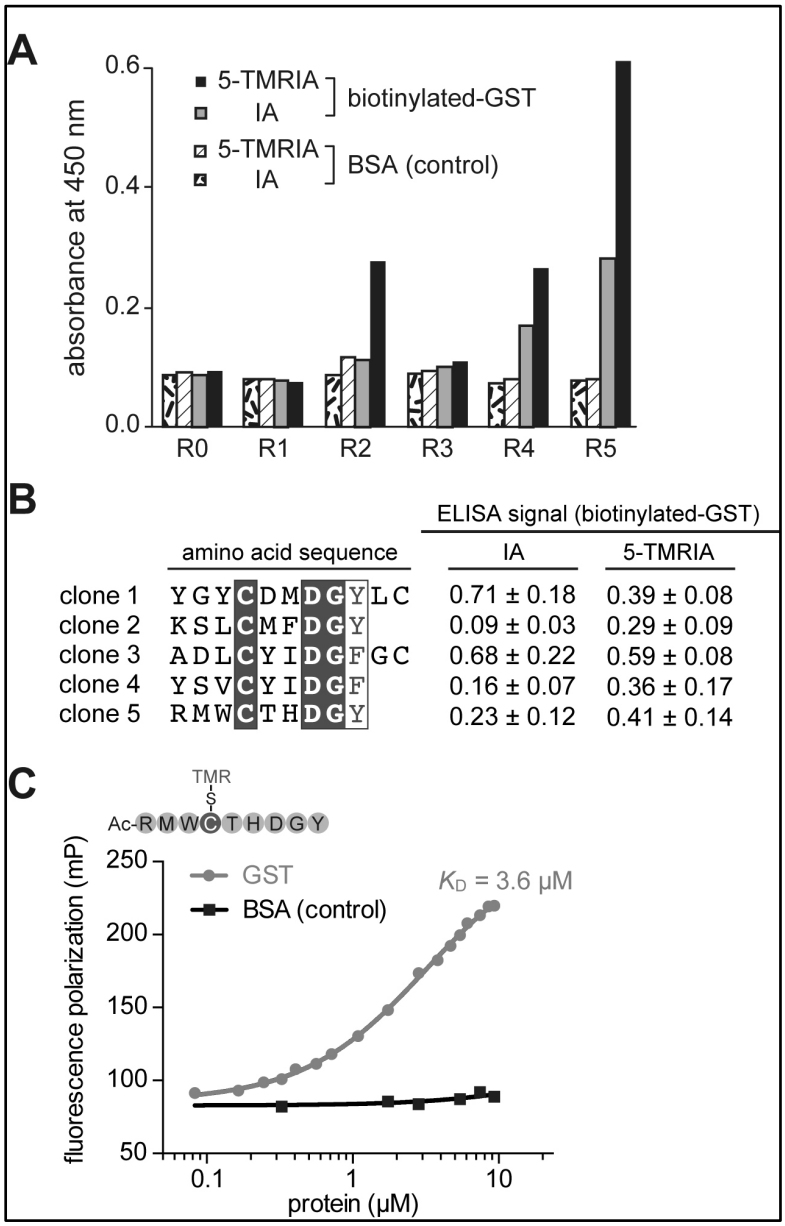


Figure 9. (**A**) ELISA of modified T7-displayed peptides against biotinlylated-GST or bovine serum albumin (BSA). T7 phage polyclones after each round of biopanning were modified with iodoacetamide (IA) or 5-TMRIA, and then subjected to ELISA. BSA was used as a negative control to examine non-specific binding. (**B**) Sequence alignment of GST-binding peptides of randomly chosen T7 clones from phage pool after 5 rounds of biopanning. Consensus sequences are highlighted. For the alignment, ESPript program[5] was used. ELISA signal intensities are shown in right. (**C**) Determination of GST-binding affinity of the TMR-conjugated Ac-R-M-W-C-T-H-D-G-Y-OH peptide by FP assay. The circles and squares indicate the polarizations (mP) of the peptide in the presence of various concentrations of GST or BSA, respectively.

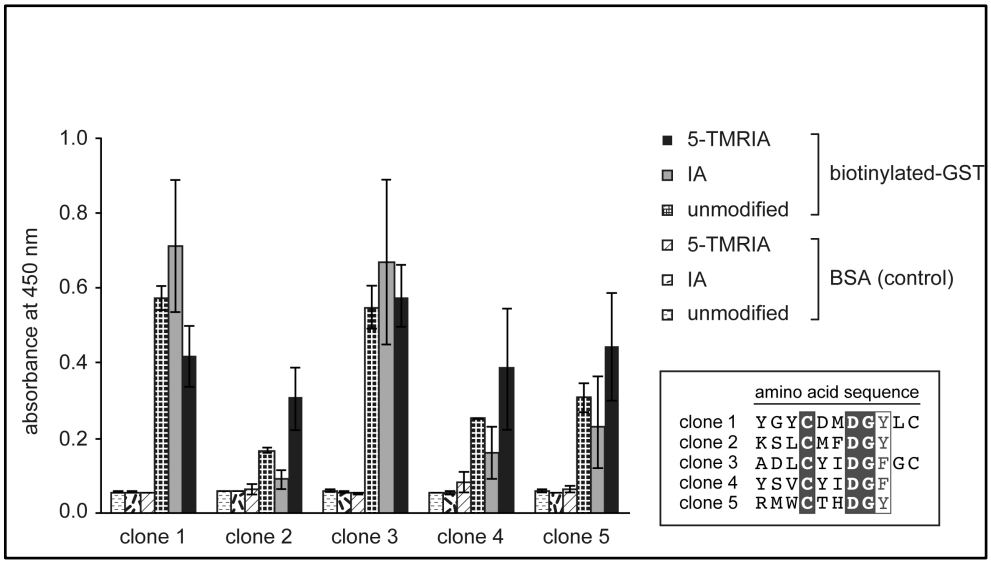


Figure 10. ELISA analysis by using T7 phage clones that randomly chosen from phage pool after 5th biopanning. Peptide sequences displayed on the phage are shown in right panel (clones 1 to 5). The graph summarizes the results of three independent experiments. Error bars represent standard deviations. IA and BSA represent iodoacetamide and bovine serum albumin, respectively.

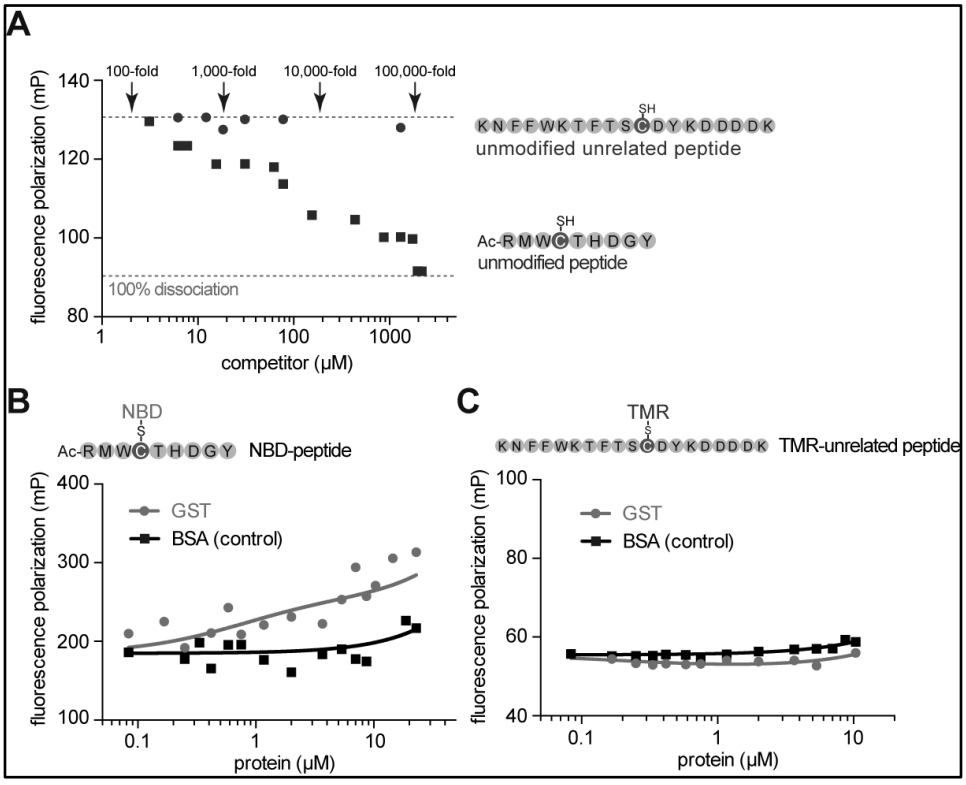


Figure 11. Fluorescence polarization assay by using negative control peptides. (**A**) Competitive binding assay. Unmodified peptides were mixed with TMR-conjugated peptide-GST complex in the presence of dithiothreitol (2 mM) in phosphate-buffered saline. The molar ratio of the competitor to TMR-conjugated peptide is represented in the upper part. (**B**) Binding of nitrobenzofurazan (NBD)-conjugated peptide with GST or BSA. When the TMR moiety was substituted to NBD moiety, the GST-specific binding of the peptide was drastically prevented. (**C**) Binding of TMR-conjugated unrelated peptide. The GST specific binding of the TMR-conjugate never occurred because the peptide sequence was totally different from that of the GST-binding peptide.

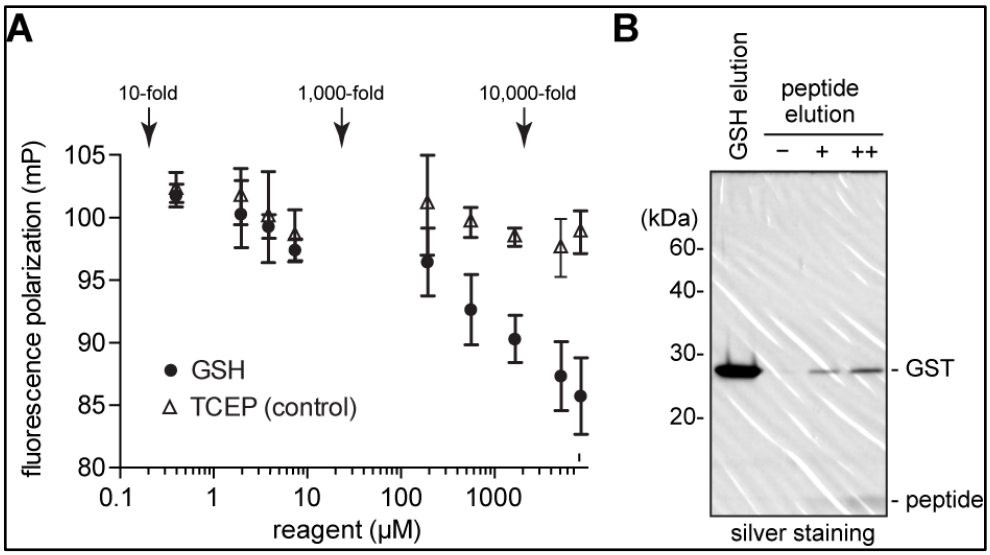


Figure 12. (**A**) Competitive binding assay. GSH was mixed with TMR-conjugated peptide-GST complex in phosphate-buffered saline. TCEP served as a mock competitor. The molar ratio of GSH to TMR-conjugated peptide is represented in the upper part. (**B**) Competitive elution of glutathione *S*-transferase (GST) from GSH-coupled sepharose.

In conclusion, I established a general and instant method of post-translational chemical modification for the T7 phage-displayed peptide library. The reactivity and specificity of the $10BASE_d$-T on the T7 phage were as excellent as those of enzymatic introduction of functional groups on proteins.[30] To my knowledge, this is the first demonstration of a construction of a T7-displayed peptide library containing non-natural structures. Numerous alkylating reagents (*e.g.,* haloacetamide derivatives) are commercially available, and such compounds can be synthesized in a simplest way.[31] I envision that the $10BASE_d$-T will be a standard method for construction of peptide-based hybrid libraries.

■ 2.3 Experimental procedures

General

All experiments were performed with commercially available reagents and kits. Note that no special materials and skills are needed. Contrary to popular belief and T7Select system manual (Merck Millipore), CsCl step gradient and ultracentrifugation are not necessary to perform all of the experiments to obtain target-specific binder.[2] For purification of T7 phage at every step by polyethyleneglycol / NaCl precipitation, I only used conventional centrifugation system which can rotate at 13,000 rpm.

Construction of T7 phage display library

FLAG-tagged T7 phage peptide library (-S-G-G-G-G-FLAG-G-S-X_3-C-X_7-C-X_3; where X represents any randomized amino acid) was constructed with T7Select10-3b (used in Figures 1A and 1D) or T7Select415-1b (used in Figure 1B) system according to the kit instructions (Merck Millipore). In brief, an oligonucleotide was synthesized in the following format: 5'-GGTGGAGGTGGCGACTACAAGGATGACGATGACAAGGGATCA $(NNK)_3TGC(NNK)_7TGT(NNK)_3$TGAAAGCTTGGA-3'. The oligonucleotide was amplified by polymerase chain reaction (PCR) using an appropriate set of primers harboring restriction sites for *Eco*RI and *Hind*III, respectively. The PCR products were purified, and then digested with *Eco*RI and *Hind*III. The DNA fragments were further purified and ligated to the vectors. The ligation products were subjected to *in vitro* packaging with T7 packaging extracts followed by infection into *Escherichia coli* BLT5403 cells. Noted that an amplification of T7 phage carrying a T7Select415-1b vector by using BLT5403 cells causes medium-high-copied peptide display (200 peptides per virion).[26] One of the T7 phage display peptide library (-S-G-G-G-X_3-C-X_7-C-X_3) was constructed in a previous study.[32] Another T7 phage display peptide library (-S-G-G-G-X_3-C-$X_{5\text{-}6}$-C-X_3) was constructed in the same manner. Quality and complexity of the phage display peptide libraries were checked by

DNA sequence analysis of PCR-amplified DNA fragments from randomly chosen phage clones. For DNA amplification, following oligonucleotides were used: 5'-CGCTAAGTACGCAATGGGCC-3' (forward primer), 5'-GTCTCAACGTTCATATCGTATGAGCG-3' (reverse primer).

Chemical modification of T7 phage via the 10BASE$_d$-T

Standard reaction condition of the 10BASE$_d$-T is the following. The reaction was carried out in 1.5 mL microcentrifuge tubes. T7 phage particles (approximately 1.0×10^{11} PFU) were dissolved in a 700 μL of phosphate-buffered saline (Nacalai, cat No. 14249-95) supplemented with 400 mM NaCl, and well suspended by sonication (vortex is also available). At this moment, the buffer was containing 1.0×10^{12} (T7 phage made by T7Select10-3b system) or 2.0×10^{13} (by T7Select415-1b system) molecules of the T7-displyed peptides. After centrifugation at 12,000 rpm for 5 minutes at room temperature, the supernatant was mixed with neutralized tris(2-carboxyethyl)phosphine (TCEP-NaOH; final concentration of 500 μM) at 4 °C. At this time, the TCEP stock aqueous solution of pH 7 was prepared by neutralization with NaOHaq in advance. Tetramethylrhodamine-5-iodoacetamide (5-TMRIA; Molecular Probes, cat. No. T-6006) was added at a final concentration of 200 μM, and the mixture was incubated at 4 °C for 3 hours in the dark with shaking. 5-TMRIA was dissolved in dimethyl sulfoxide/water (1:1 by volume) in advance. To inactivate the unreacted 5-TMRIA, 2-mercaptoethanol was added to the mixture at a final concentration of 5 mM, and further incubated at 0 °C for several minutes. The T7 phage particles were precipitated with a mixture of polyethylene glycol 6000 and sodium chloride to final concentrations of 5% (w/v) and 0.5 M, respectively. After centrifugation, the precipitate was dissolved in an appreciate buffer.

In-gel fluorescence imaging and Western blot analysis

T7 phage particles were dissolved in 1 × sample buffer (62.5 mM tris(hydroxymethyl)aminomethane-HCl, pH 6.8, 10% glycerol, 2% SDS, 5%

2-mercaptoethanol, 0.002% bromophenol blue). The solution was incubated at 95 °C for 5 min, and then subjected to SDS-PAGE. Proteins were resolved by a 10% or a 10-20% gradient SDS–polyacrylamide gel. After electrophoresis, the TMR-conjugated proteins were visualized by in-gel fluorescence imaging using a fluoroimager (FMBIO III-SC01, Hitachi, Japan) as reported previously.[33] A band-pass filter (555 BP20) was used for the detection. A conventional gel imager excited by UV light, such as Gel Doc XRS+ (Bio-Rad), can also take the image.

Western blot analysis was performed as described previously.[34] In brief, proteins in the gel were transferred onto a polyvinylidene difuoride (PVDF) membrane (Bio-Rad), and then the membrane was subjected to blocking with bovine serum albumin. The blots were incubated with anti-FLAG M2 mouse monoclonal antibody (1/1,000 dilution; Sigma Aldrich), followed by incubation with anti-mouse IgG horseradish peroxidase-conjugated secondary antibody (1/5,000 dilution Cell Signaling Technology). After several washes, the blots were incubated with ECL plus reagent (GE Healthcare Life Sciences), and detected using a ChemiDoc XRS+. Image contrast and brightness were adjusted in Photoshop CS4 (Adobe).

Site-specific cleavage of T7-displayed peptides by enterokinase

Site-specific cleavage of peptide-fused gp10 was performed as previously described.[31] In brief, non-denatured T7 phage particles were suspended in a reaction buffer (20 mM tris(hydroxymethyl)aminomethane-HCl, pH 7.4, 50 mM NaCl, 2 mM $CaCl_2$) in the presence or absence of 0.1 units of recombinant enterokinase (Merck Millipore), and incubated at 37 °C for 16 hours. The reaction was stopped by an addition of 4 × sample buffer.

Mass spectrometric analysis

For mass spectrometric analysis, a PAGE gel was stained with a rapid stain CBB kit (Nacalai, Japan). The stained protein bands were excised from the gel. Proteins in the gel were reduced with 25 mM dithiothreitol at 65 °C for 10 min, and then alkylated with

55 mM iodoacetamide at room temperature for 60 min in the dark. Digestion was carried out with modified trypsin (Promega, Madison, WI) or lysyl endopeptidase (Wako, Japan) at 37 °C overnight. The resulting peptides were analyzed using an Agilent 1100 HPLC system (Agilent Technologies) equipped with a C_{18} reverse-phase column (Hypersil GOLD, 2.1 × 100 mm, Thermo Fisher Scientific) connected to a LCQ-Fleet mass spectrometer. The peptides were separated using a 0-50% gradient of acetonitrile containing 0.1% formic acid during 40 min at a flow rate of 300 μL per a minute, and then eluted peptides were directly sprayed into the mass spectrometer. The mass spectrometer was operated in the data-dependent mode and externally calibrated. Survey MS scans were acquired in the 400-2000 *m/z* range. Multiply charged ions of high intensity per scan were fragmented with CID in the ion trap. A dynamic exclusion window was applied within 30 seconds. All tandem mass spectra were collected using normalized collision energy of 35%. Data were acquired and analyzed with Xcalibur software (Thermo Fisher).

GST expression and biotinylation followed by biopanning

For preparation of GST protein, pGEX-4T-3 vector (GE Healthcare) was introduced into *E. coli* BL21 (DE3) strain. Transformants were precultured overnight at 37°C in 10 mL of LB medium supplemented with 100 μg/mL ampicillin, and then transferred to a 200 mL of fresh medium. After incubation for 2 hours at 37 °C, isopropyl β-D-1-thiogalactopyranoside was added at a final concentration of 0.2 mM, and the cells were further cultured for 2 hours. The cells were harvested, and suspended in B-PER protein extraction reagents (Thermo Scientific) supplemented with lysozyme. After incubation for 1 hour at 4 °C, the sample was cleared by centrifugation at 20,000 × *g* (13,000 rpm) for 10 min at 4 °C. Supernatant was incubated with glutathione sepharose 4B (GE Healthcare). After several washing with tris-buffered saline supplemented with 0.5 % Triton X-100, GST protein was eluted with glutathione elution buffer (50 mM PIPES-KOH, pH 7.4, 100 mM NaCl, 1 mM dithiothreitol, and 50 mM reduced glutathione). The eluted protein was desalted by ultrafiltration, and then

subjected to biotinylation with a biotin labeling kit (DOJINDO, Japan) followed by purification. The biotinylation of the GST was confirmed by Western blotting. Purity of the biotinlylated-GST was estimated to be above 95%. 20 pmol of the biotinlylated-GST was immobilized on streptavidin-coupled Dynabeads (Invitrogen).

For biopanning, approximately 8.4×10^{10} PFU of T7 phage display peptide libraries (-S-G-G-G-X_3-C-$X_{5\text{-}7}$-C-X_3; X represents any randomized amino acid) were modified via the $10BASE_d$-T. To eliminate T7 phage that bound to the Dynabeads, the modified T7 phage display peptide library was dissolved in selection buffer (phosphate-buffered saline supplemented with 1% Triton X-100 and 1% bovine serum albumin), and pre-incubated with the beads for 2 hours at 4 °C. Then, the supernatant was further incubated with the biotinylated-GST immobilized Dynabeads for 2 hours at 4 °C. The beads were washed three times with 200 μL of the selection buffer, and the GST-bound phage was directly infected and amplified with *E. coli* BLT5403 cells. Stringent conditions were stepwisely applied to each round by shortening the binding time and by increasing the washing frequency. After 5th rounds of the panning, randomly chosen T7 phage clones were subjected to DNA sequencing. Overview of the biopanning in this study was shown in Figure 8.

Enzyme-linked immunosorbent assay (ELISA)

830 pmol of streptavidin (Promega) was dissolved in phosphate-buffered saline (PBS), and immobilized on each well of a 96 well immunoplate (Nunc MaxiSorp, Thermo Scientific). After washing with PBS, surface of the wells were coated with 1% (w/v) bovine serum albumin in PBS supplemented with 0.05% Tween-20 at 4°C overnight. 5 pmol of biotinylated-GST was immobilized on it, and unbound protein was washed. Approximately 2.0×10^{10} PFU of the T7 phage was dissolved in tris-buffered saline supplemented with 0.5% Triton X-100, and applied to the well plate. The plate was incubated for 1 hour at 25 °C with shaking by using a maximizer (MBR-022UP, Taitec, Japan), and then washed three times with tris-buffered saline supplemented with 0.5% Triton X-100. The bound phage was incubated with T7 tail fiber monoclonal

antibody (1:5,000 dilution, Merck Millipore) and anti-mouse IgG HRP-linked antibody (1:5,000 dilution, Cell Signaling) for 1 hour at 25 °C with shaking. After washing, o-phenylenediamine dihydrochloride substrate (SigmaFast OPD, Sigma Aldrich) was added, and the absorbance was quantified using a microplate reader equipped with a 450 nm band-pass filter (Bio-Rad).

GST-binding peptide synthesis and fluorescence polarization assay

A peptide (acetyl-R-M-W-C-T-H-D-G-Y-OH) was synthesized and characterized by HPLC and ESI-TOF-MS by GenScript Corp (USA). A mock peptide (H_2N-K-N-F-F-W-K-T-F-T-S-C-D-Y-K-D-D-D-D-K-OH) was synthesized and used in a previous study.[7] Purity of the peptide was estimated to be above 95%. For the conjugation of tetramethylrhodamine (TMR) or nitrobenzofurazan (NBD), alkylating reagent and neutralized TCEP (pH 7) were mixed with the peptide (100 μM) in a phosphate buffer (20 mM phosphate-KOH, pH 7.4) at final concentrations of 150 μM and 500 μM, respectively. 5-TMRIA (Tetramethylrhodamine-5-Iodoacetamide; cat. No. T-6006) and IANBD amide (N,N'-Dimethyl-N-(iodoacetyl)-N'-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine; cat. No. D-2004) were purchased from Molecular Probes. The mixture was incubated overnight at 37 °C in the dark with shaking. After addition of formic acid at a final concentration of 1%, the TMR-conjugated peptide was purified with reverse-phase HPLC (Shimadzu, Japan) equipped with a XTerra Prep MS C_{18} column (10 × 50 mm, Waters). The peptide was separated using a 0-100% linear gradient of acetonitrile containing 0.1% trifluoroacetic acid during 12 min at a flow rate of 4 ml per a minute. Fractions containing the TMR-conjugate peptide Ire lyophilized and then dissolved in dimethyl sulfoxide. Peptide concentrations were calculated by absorption peak at 280 nm. Characterization of the peptides was performed by LC-MS (see above; Fig. S1). Purity was estimated to be above 90%.

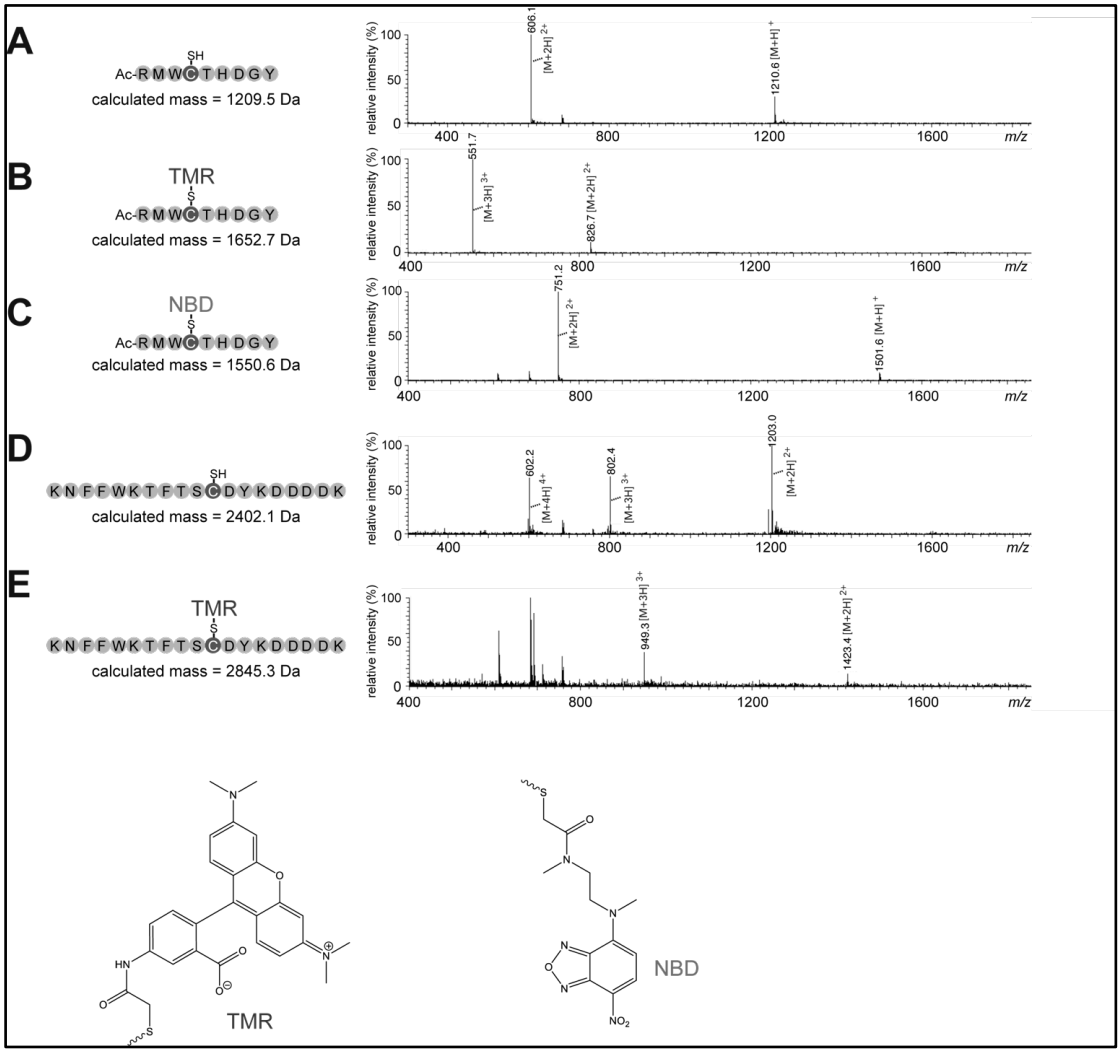


Figure S1. LC-MS analysis of chemically synthesized peptides. (**A**) unmodified Ac-R-M-W-C-T-H-D-G-Y-OH peptide (equivalent to the T7 phage clone 5 in Fig. 3B and S7). (**B**) TMR-conjugated peptide. (**C**) NBD-conjugated peptide. (**D**) unmodified unrelated peptide.[7] (**E**) TMR-conjugated unrelated peptide. Chemical structures of TMR and NBD are shown in below.

Fluorescence polarization was measured with a HYBRID-3000ES (Photoscience, Japan) equipped with appropriate filters (TMR: Ex. 535/540 nm and Em. 570 nm, NBD: Ex. 480 nm and Em. 530 nm). The fluorophore-conjugated peptide (4 pmol, 20 nM) was incubated with various concentrations of GST or BSA in phosphate-buffered saline at 30°C. Klotz plot was generated by GraphPad Prism software 6.0 (GraphPad Software, San Diego, CA), and the sigmoid curve was fitted with non-linear least squares analysis to obtain the dissociation constant.

■ 2.4 References

[1] C. J. Hipolito and H. Suga, *Curr. Opin. Chem. Biol.*, 2012, **16**, 196-203.

[2] T. Kawakami and H. Murakami, *J. Nucleic Acids*, 2012, **2012**, 713510.

[3] A. C. Forster, Z. Tan, M. N. Nalam, H. Lin, H. Qu, V. W. Cornish and S. C. Blacklow, *Proc. Natl. Acad. Sci. U. S. A.*, 2003, **100**, 6353-6357.

[4] K. Josephson, M. C. Hartman and J. W. Szostak, *J. Am. Chem. Soc.*, 2005, **127**, 11727-11735.

[5] P. Gouet, E. Courcelle, D. I. Stuart and F. Metoz, *Bioinformatics*, 1999, **15**, 305-308.

[6] M. Sisido and T. Hohsaka, *Bull. Chem. Soc. Jpn.*, 1999, **72**, 1409-1425.

[7] A. Angelini and C. Heinis, *Curr. Opin. Chem. Biol.*, 2011, **15**, 355-361.

[8] S. Ng, M. R. Jafari and R. Derda, *ACS Chem. Biol.*, 2012, **7**, 123-138.

[9] J. Rodriguez, N. Gupta, R. D. Smith and P. A. Pevzner, *J. Proteome Res.*, 2008, **7**, 300-305.

[10] G. P. Smith and V. A. Petrenko, *Chem. Rev.*, 1997, **97**, 391-410.

[11] L. Jespers, T. P. Bonnert and G. Winter, *Protein Eng. Des. Sel.*, 2004, **17**, 709-713.

[12] C. Heinis, T. Rutherford, S. Freund and G. Winter, *Nat. Chem. Biol.*, 2009, **5**, 502-507.

[13] V. Baeriswyl, S. Calzavarini, C. Gerschheimer, P. Diderich, A. Angelillo-Scherrer and C. Heinis, *J. Med. Chem.*, 2013, **56**, 3742-3746.

[14] A. Angelini, L. Cendron, S. Chen, J. Touati, G. Winter, G. Zanotti and C. Heinis, *ACS Chem. Biol.*, 2012, **7**, 817-821.

[15] G. A. Barkocy-Gallagher, J. G. Cannon and P. J. Bassford, Jr., *J. Biol. Chem.*, 1994, **269**, 13609-13613.

[16] E. H. Manting and A. J. Driessen, *Mol. Microbiol.*, 2000, **37**, 226-238.

[17] K. Fukunaga and M. Taki, *J. Nucleic Acids*, 2012, **2012**, 295719.

[18] K. Arai, H. Tsutsumi and H. Mihara, *Bioorg. Med. Chem. Lett.*, 2013, **23**, 4940-4943.

[19] H. Houshmand, G. Froman and G. Magnusson, *Anal. Biochem.*, 1999, **268**, 363-370.

[20] L. R. Krumpe, A. J. Atkinson, G. W. Smythers, A. Kandel, K. M.

Schumacher, J. B. McMahon, L. Makowski and T. Mori, *Proteomics*, 2006, **6**, 4210-4222.

[21] A. Ionel, J. A. Velazquez-Muriel, D. Luque, A. Cuervo, J. R. Caston, J. M. Valpuesta, J. Martin-Benito and J. L. Carrascosa, *J. Biol. Chem.*, 2011, **286**, 234-242.

[22] U. T. Ruegg and J. Rudinger, *Methods Enzymol.*, 1977, **47**, 111-116.

[23] R. J. Simpson, *Proteins and Proteomics: A Laboratory Manual Cold Spring Harbor Laboratory Press.*, 2003.

[24] I. Rentero Rebollo and C. Heinis, *Methods*, 2013, **60**, 46-54.

[25] A. Rosenberg, K. Griffin, F. W. Studier, M. McCormick, J. Berg, R. Novy and R. Mierendorf, *inNovations*, 1996, **6**, 1-6.

[26] K. D. Wittrup and G. L. Verdine, *Methods Enzymol.*, 2012, **503**, xiii-xiv.

[27] K. Somers, P. Stinissen and V. Somers, *Proteomics*, 2011, **11**, 2550-2554.

[28] B. Wu and D. Dong, *Trends Pharmacol. Sci.*, 2012, **33**, 656-668.

[29] J. D. Hayes, J. U. Flanagan and I. R. Jowsey, *Annu. Rev. Pharmacol. Toxicol.*, 2005, **45**, 51-88.

[30] M. Taki, M. Shiota and K. Taira, *Protein Eng. Des. Sel.*, 2004, **17**, 119-126.

[31] T. Hamamoto, M. Sisido, T. Ohtsuki and M. Taki, *Chem. Commun. (Camb)*, 2011, **47**, 9116-9118.

[32] T. Hatanaka, S. Ohzono, M. Park, K. Sakamoto, S. Tsukamoto, R. Sugita, H. Ishitobi, T. Mori, O. Ito, K. Sorajo, K. Sugimura, S. Ham, and Y. Ito. *J. Biol. Chem.*, 2012, **287**, 43126.

[33] J. Kawaguchi, K. Maejima, H. Kuroiwa, and M. Taki. *FEBS Open Biol.*, 2013, **3**, 252.

[34] K. Fukunaga, T. Kudo, A. Toh-e, K. Tanaka, and Y. Saeki. *Biochem. Biophys. Res. Commun.*, 2010, **396**, 1048.

CHAPTER 3

Construction of a salicylic acid-conjugated peptide library and selection of streptavidin-binders

■ Abstract

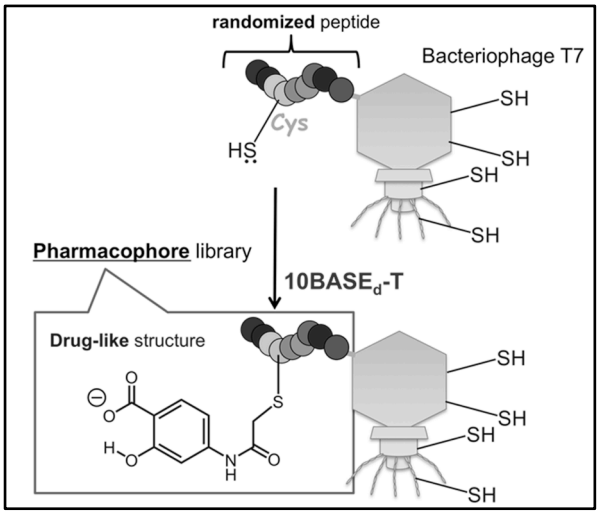
I have achieved site-specific conjugation of several haloacetamide derivatives into designated cysteines on bacteriophage T7-displayed peptides, which are fused to T7 capsid protein gp10. This easiest gp10 based-thioetherification (10BASE$_d$-T) undergoes almost quantitatively like a click reaction without side reaction or loss of phage infectivity. The post-translational modification yield, as well as the site-specificity, is quantitatively analyzed by a fluorescent densitometric analysis after gel electrophoresis. The detailed structure of the modified peptide on phage is identified with tandem mass spectrometry. Construction of such peptide-based phage library displaying non-natural structures will be useful for future drug discovery. For this aim, I propose a novel concept of pharmacophore generation from a drug-like molecule (i.e., salicylic acid) conjugated with randomized peptide. By using the hybrid library, streptavidin-specific binders are isolated through 4 rounds of biopanning.

■ 3.1 Introduction

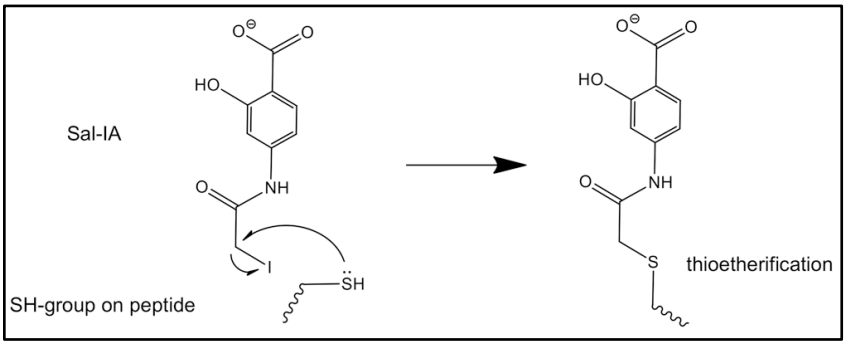
Recently, post-translational chemical modification of bacteriophage (referred to hereafter as phage)-displayed peptides is attracting attention for drug discovery.[1, 2] A pioneer work of the non-natural peptide/protein library construction on phage was reported in 2004; a fluorogenic biosensor was developed by conjugating a fluorophore with a designated Cys at the antigen-binding site of antibody library on the phage.[3] Until now, multiple research groups have reported construction of non-natural peptide libraries by the post-translational chemical modifications of M13 phage-displayed peptides.[2, 4-9] Another type of phage, T7, is also used for phage display,[10, 11] and superior to the M13 phage in view of bias and handling.[12] Very recently, I have constructed a non-natural peptide library by the post-translational chemical modification of T7 phage-displayed peptides, namely gp10 based-thioetherification (10BASE$_d$-T).[13] The 10BASE$_d$-T is carried out in one-pot without side reactions or loss of phage infectivity; the reaction efficiency and site/position specificity of the 10BASE$_d$-T on the T7 phage were as excellent as those of (chemo)enzymatic or click introduction of functional groups on proteins. By using a tetramethylrhodamine (TMR)-conjugated library via the 10BASE$_d$-T, glutathione *S*-transferase specific-binders have been discovered.

A potential limitation of this library is that the TMR moiety is too bulky and hydrophobic, which may cause non-specific binding or aggregation of the binders. Thus, I envision that conjugation of a water-soluble and small drug-like molecule, instead of TMR, to a peptide library would increase the possibility to discover target-specific binders. Here I attempt a novel concept of a pharmacophore generation by conjugation of a drug-like molecule to a randomized library peptide via the 10BASE$_d$-T (Scheme 1). As the model drug-like molecule, commercially available 4-iodoacetamidosalicylic acid (Sal-IA) was used because it is one of the smallest pharmaceutical molecules possessing both hydrophobic benzene ring and hydrophilic hydroxyl / carboxyl groups. These groups may potentially interact with various proteins through hydrophobic / pi

interactions and hydrogen bonding,[14] and seldom form aggregates.[15] If surroundings of the drug-like molecule are optimized for interaction with target biomolecules of interest, novel specificity and affinity would be generated. Thus, I constructed the artificial peptide library possessing a salicylic acid (Sal) moiety via the 10BASE$_d$-T.



Scheme 1. Construction of a peptide-fused pharmacophore library with a model drug-like (i.e., salicylic acid; Sal) core structure via the 10BASE$_d$-T.



Scheme 2. Thioetherification of SH-group on peptide by S_N2 reaction.

■ 3.2 Results and discussion

For the optimization of site-specific introduction of Sal group via the 10BASE$_d$-T, I mixed Sal-IA with various molar concentrations and a T7-displayed model peptide (1.0×10^{11} PFU of the T7 phage) in 700 μL of phosphate-buffered saline (pH 7.4) supplemented with 500 μM tris(2-carboxyethyl)phosphine (TCEP) and 400 mM NaCl at 4 °C. After 3 hours reaction, the peptide was further treated with 5-idodoacetamide-fluorescein (FL-IA; 200 μM) for 3 hours at 4 °C. The latter reaction with fluorescent FL-IA blocks all the unreacted SH-groups on the T7 phage-displayed peptide after the conjugation with Sal-IA. My study demonstrated that fluorescent tetramethylrhodamine-iodoacetamide is conjugated to at least 95% of T7 phage-displayed peptide.[13] Similarly, I confirmed that FL-IA was conjugated to at least 95% of the displayed peptide when 200 μM of FL-IA was used for the modification (data not shown). Thus, I can indirectly estimate conversion yield of the Sal-IA conjugation by fluorescent densitometric analysis (Fig. 1A). After the 10BASE$_d$-T, whole T7 phage proteins were subjected to sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE), followed by in-gel fluorescence imaging. When Sal-IA was absent in the reaction mixture, a single fluorescent band could be seen at an appropriate molecular weight (*ca.,* 44 kDa) of the peptide-fused gp10 (Fig. 1B; upper panel, lane 2). This indicates that the alkylation with FL-IA exclusively occurred at the peptide-fused gp10. Note that neither protein components for the infection nor other T7 phage molecules were included in the reaction.[13] When the concentration of Sal-IA was increased, the fluorescent band disappeared. This indicates that the designated Cys on the displayed peptide had already reacted with Sal-IA, and FL-IA no longer reacted with the peptide. From the densitometric analysis, the optimal molar concentration of Sal-IA was around 800 μM; almost all the designated Cys on the peptide reacted with Sal-IA (Fig. 1C). Thus, I successfully established a quantification method of the 10BASE$_d$-T reaction in a visible manner, even if the introduced non-natural molecule does not possess any chromophores. Recently, Derda and co-workers demonstrated a

compatible quantification method, namely the biotin capture assay, to determine the chemical modification yield on M13 phage-displayed peptides.[2, 7] In this method, biotin is once conjugated to the M13 phage-displayed peptides. Then, the biotin-conjugated M13 phage is captured by streptavidin-conjugated affinity beads and subjected to a plaque assay. This method might be useful when the amount of the modified phage is extremely limited. If it is sufficient, my quantification method shown above is rapid and straightforward.

Under the optimized modification conditions, I examined the infectivity of the Sal-conjugated T7 phage peptide library by plaque assay and found that the modified T7 phage retained its infectivity (Fig. 2). This suggests that the T7 phage is an excellent platform for post-translational modifications.

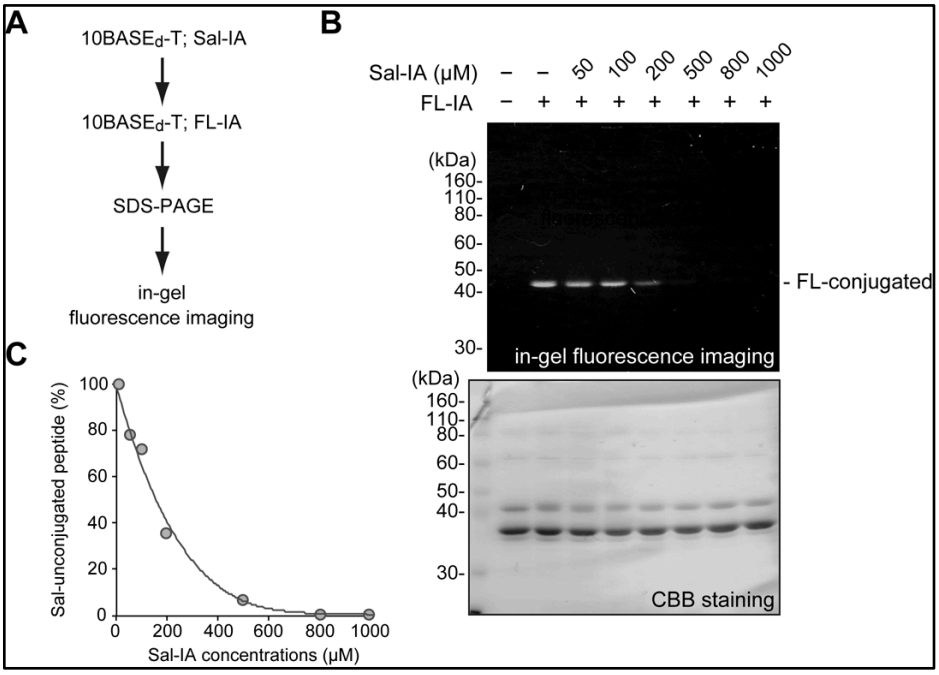


Figure 1. Optimization of Sal-IA concentration. (**A**) Procedure of the optimization. A T7 phage-displayed model peptide was modified via the 10BASE$_d$-T with various molar concentrations of Sal-IA under standard conditions (described in Materials and Methods). After the Sal-conjugation, the peptide was further modified via the 10BASE$_d$-T with 200 μM of FL-IA. Equal amounts of phage proteins were subjected to SDS-PAGE followed by fluorescence imaging. Sal-IA and FL-IA represents salicylic acid- iodoacetamide and fluorescein-iodoacetamide, respectively. (**B**) In-gel fluorescence imaging after the gel

electrophoresis (upper panel). Total T7 phage proteins were stained with coomassie brilliant blue (CBB) (lower panel). (C) Percentages of Sal-unconjugated peptide in each concentration of Sal-IA.

To achieve more precise characterization, I performed a mass spectrometric analysis of the Sal-conjugated peptide. MS analysis of the phage-displayed peptide is challenging because small amounts of peptides are obtained per batch.[2] Similar to M13 phage-displayed peptides fused to a minor coat protein pIII,[16] relatively few copies (*ca.,* 10 molecules) of peptides are displayed per single T7 phage virion when a mid-copy phage display vector (T7Select10) is used.[17] These protein amounts are nearly at the detection limit for MS-based characterization of the peptide.[2] To bypass this problem, I generated a model T7 phage[13] carrying approximately 200 molecules of the peptides per single virion by using a high-copy vector (T7Select415).[18] In my study, I successfully analyzed TMR-conjugated peptides on the model T7 phage by conventional LC-MS/MS.[13] After the 10BASE$_d$-T with Sal-IA followed by SDS-PAGE, the peptide-fused gp10 band around 40 kDa in the gel was excised and digested with trypsin. The resulting peptide fragments were analyzed by LC-MS/MS. Two chromatographic peaks at 300-400 nm (Sal absorption) were detected (data not shown), and each of them was identified as the Sal-conjugated T7 phage-displayed peptide by tandem mass spectrometry (Fig. 3). Also these data suggest that two Sal molecules were conjugated to the two designated Cys on T7 phage-displayed peptides. At the same time, most of the other peptide fragments derived from gp10 were identified by peptide mass fingerprinting based on MS/MS ion search (data not shown). Very recently, mass spectrometric analysis of the modified M13 phage-displayed peptide was reported by using high-performance MS systems.[19, 20] In future studies, not only qualitative but also quantitative mass spectrometric study of the modified phage-displayed peptide might be available.

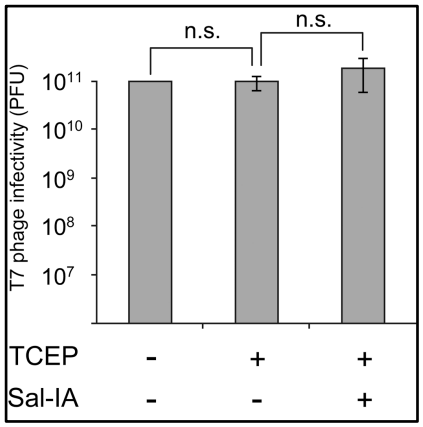


Figure 2. Infectivity of Sal-conjugated T7 phage library. A T7 phage library (-S-G-G-G-X_3-C-X_6-C-X_3; X represents any randomized amino acid) was treated with or without salicylic acid-iodoacetamide (Sal-IA; 800 μM) in the presence of TCEP (500 μM) under standard conditions (see Experimental Section). The number of plaque forming units (PFU) was determined by a serial dilution method and plaque assay. The graph summarizes the results of three independent experiments. Error bars represent standard deviations. Statistical analysis was performed by unpaired Student's *t*-test. n.s., not significant (*p* values = 0.40 [left] and 0.22 [right], respectively).

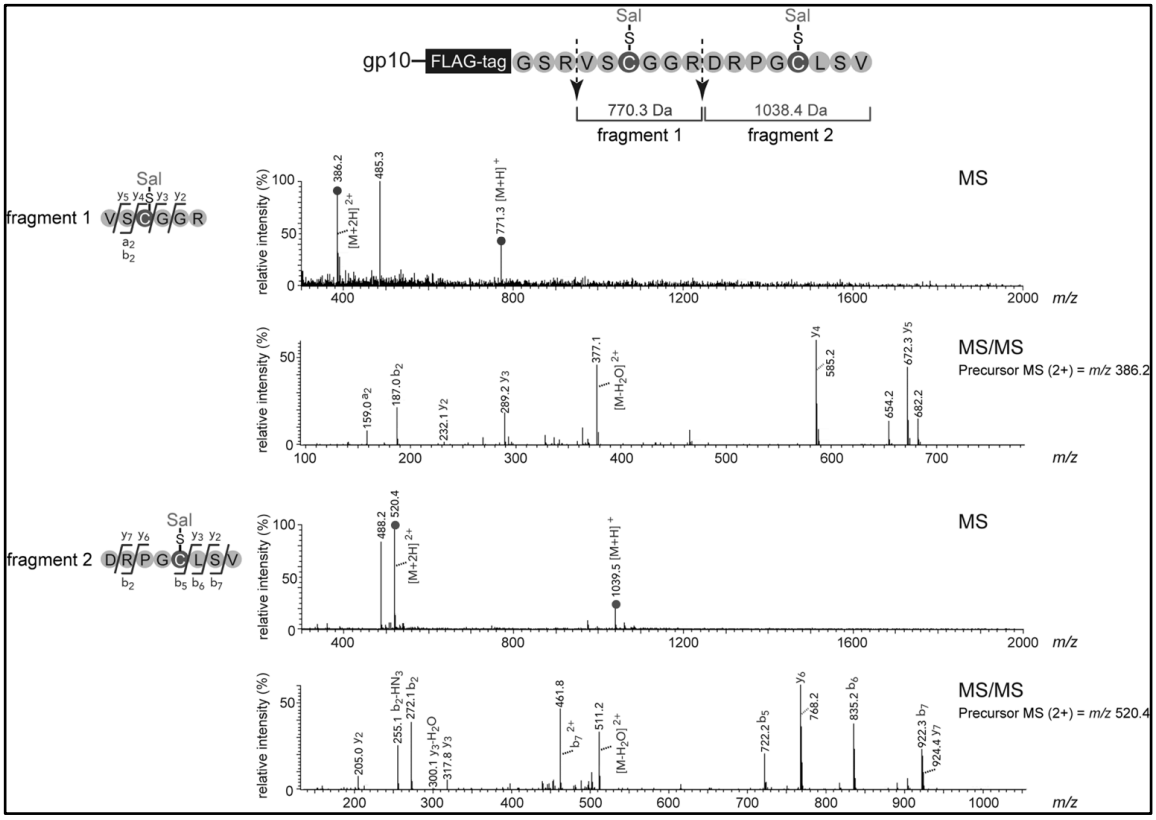


Figure 3. Introduction of Sal onto T7 phage-displayed peptide via the 10BASE$_d$-T. Here, a model T7 phage carrying a high-copy vector was used. After the 10BASE$_d$-T followed by SDS-PAGE, Sal-conjugated peptide-fused gp10 was excised and subjected to in-gel trypsinization followed by conventional LC-MS/MS analysis. Note that trypsin could not cleave before proline.[13] MS and MS/MS spectra correspond to the trypsinized two peptide fragments possessing the Sal moiety.

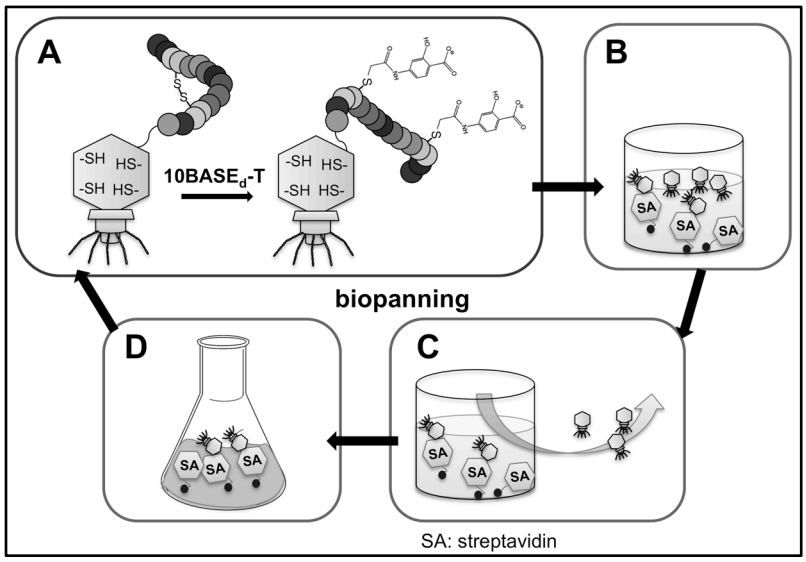


Figure 4. Schematic diagram of biopanning used in this study. (**A**) Construction of Sal-conjugated peptide library on T7 phage through the 10BASE$_d$-T. (**B**) Incubation of the phage display peptide library with immobilized streptavidin. (**C**) Washing of unbound phage. (**D**) Amplification of the streptavidin-bound phage for subsequent rounds of biopanning.

I next attempted to find target-specific binders from the Sal-conjugated peptide library as shown in Fig. 4. In advance of biopanning, a T7 phage library carrying X_3-C-X_6-C-X_3 peptides (where X represents any amino acid), which had been generated from the mid-copy vector (T7Select10), were modified through the 10BASE$_d$-T. To include the possibility that two alkylated cysteines may improve affinity toward the target protein, I conjugated two Sal-groups into a single library peptide. Four rounds of biopanning were performed against streptavidin as a model target protein. Enrichment of the streptavidin-binders were confirmed by plaque assay after the washing step in

biopanning; the population of streptavidin-bound T7 phage was increased through biopanning (Fig. 5A). Binding specificity to streptavidin was confirmed by enzyme-linked immunosorbent assay (ELISA). To examine whether the Sal but not the amide moiety is required for the binding, Sal-IA-treated and iodoacetamide (IA)-treated T7 phage were subjected to ELISA in parallel. After 4 rounds of biopanning, the Sal-conjugated T7 phage-displayed peptides showed the strongest binding to streptavidin (Fig. 5B), suggesting that the Sal moiety of the peptides played a crucial role in interaction with the target. Eight of the T7 phage monoclones were randomly chosen from the phage pool. Three of the streptavidin-bound monoclones were subjected to DNA sequencing analysis, whereas five of the clones did not bind to streptavidin. Two of the positive clones had the same sequence (clone 7; -A-M-W-C-Q-Y-H-P-Q-N-C-Y-K-M), and one was different (clone 2; -R-I-V-C-V-Q-H-P-Q-F-C-Q-Y) (Fig. 5C). I also found that such clones had a consensus sequence of C*-X-X-H-P-Q-X-C* (C* and X represent alkylated Cys and any amino acid, respectively) (Fig. 5C, left panel), which contains the known streptavidin-binding sequence (H-P-Q).[21, 22] However, this peptide on the T7 phage exclusively bound to streptavidin only when it was modified with Sal-IA. This means that the Sal moiety and the surrounding consensus peptide cooperatively enhanced the binding ability toward streptavidin, to generate a novel pharmacophore (Fig. 5C).

To determine the binding affinity of the peptide to streptavidin by using a fluorescence polarization (FP) assay, I chemically synthesized a fluorescent peptide possessing the sequence of clone 7 (K-5/6-FAM-A-M-W-C*-Q-Y-H-P-Q-N-C*-Y-K-M-NH_2; C* represents Sal-conjugated Cys). The synthesized linear peptide bound to streptavidin in a protein-specific manner (Fig. 6A, left panel), which is consistent with a previous report that HPQ-containing peptide does not bind to non-glycosylated forms of chicken avidin (NeutrAvidin®).[23] The dissociation constant (K_D) was estimated to be 180 nM. Usually, linear peptides bind to a target with a lower affinity than cyclic ones, because the latter rigid structures minimize conformational entropy loss associated with the binding.[12, 24] Indeed,

H-P-Q-containing cyclic peptides bind to streptavidin with a higher affinity.[21, 25] Nevertheless, the affinity of Sal-conjugated linear peptide was almost the same as that of the H-P-Q-containing cyclic peptide.[25] To my knowledge, there is no streptavidin-binding linear peptide with submicromolar affinity; exceptionally, H-P-Q-containing 38-mer linear *protein*, SBP-tag, binds to streptavidin with high affinity (i.e., 2.5 nM).[26] On the other hand, mock (Sal-unconjugated) peptide bound to streptavidin with a roughly 10-fold lower affinity under reducing conditions (Fig. 6A, right panel). This again suggests that the excellent pharmacophore was generated only when Sal was surrounded by the appropriate peptide.

H-P-Q-containing peptides are usually biotin-mimetics,[21] thus I examined whether the Sal-conjugated peptide binds to streptavidin in the same manner. As expected, interaction of the Sal-conjugated peptide with streptavidin was fully disrupted by biotin (Fig. 6B), suggesting that the peptide binds to the biotin-binding site of streptavidin.

In conclusion, site-specific conjugation of haloacetamide derivatives to the designated Cys on bacteriophage T7-displayed peptides was achieved. This underwent almost quantitatively without side reaction. The structure of the modified peptide on phage was identified with tandem mass spectrometry, and the conjugation yield was estimated by SDS-PAGE followed by fluorescence imaging in a rapid and universal manner. Generation of a novel pharmacophore by conjugation of a drug-like molecule to fully randomized peptide on T7 phage was also demonstrated. Recently, optimization of a pharmacophore by conjugation with library peptide via the mRNA-display and M13 phage display techniques were reported.[8, 29] In both cases, the pharmacophore molecule has already been known to bind a target protein, and the binding ability was *improved* by *in vitro* selection. In contrast, I demonstrated that even optimization of a small drug-like molecule, which is *never* known to bind to a model target protein, could generate a novel pharmacophore. I envision that computer assisted *de novo* designing,[30] data mining from broad public databases,[31] and/or docking simulation of the small drug-like molecule[31, 32] followed by optimization of its surroundings by peptide via the 10BASE$_d$-T will be a general technology for drug discovery.

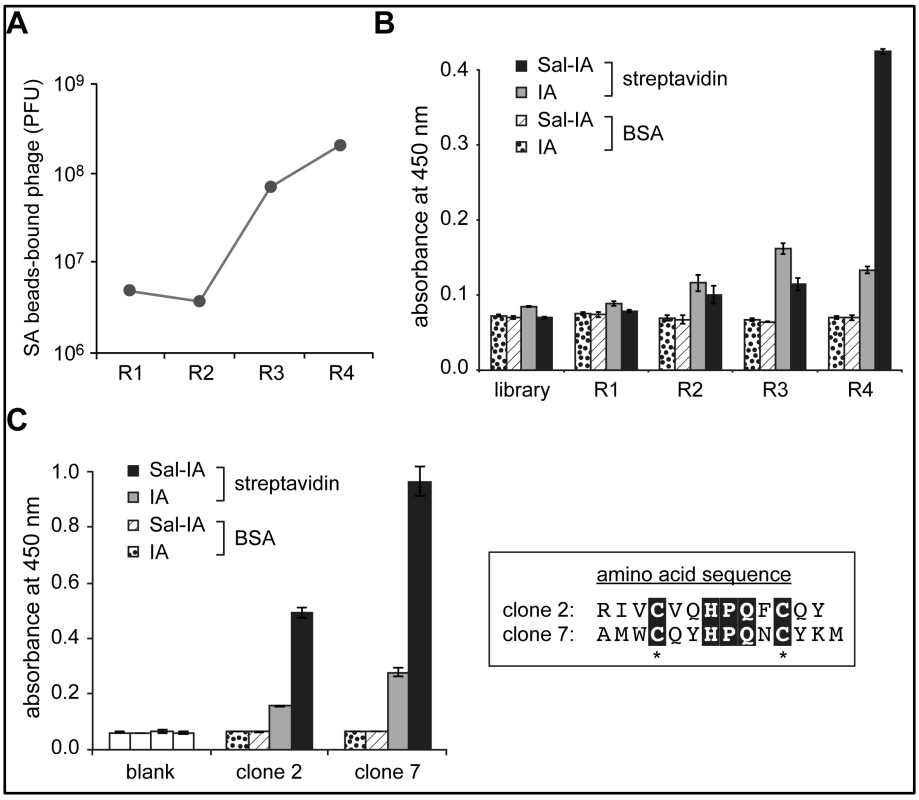


Figure 5. (**A**) Enrichment of streptavidin (SA)-coupled beads-bound T7 phage through biopanning. PFU (plaque forming units) and R indicate the number of the beads-bound T7 phage and rounds of biopanning, respectively. (**B**) ELISA of modified T7 phage polyclones against streptavidin or bovine serum albumin (BSA). BSA was used as a negative control to examine non-specific binding. Sal-IA and IA indicate salicylic acid-iodoacetamide and iodoacetamide, respectively. Error bars represent standard deviations of three independent experiments. **(C)** ELISA of two types of T7 phage monoclones randomly chosen from the phage pool after 4 rounds of biopanning. The peptide sequences displayed on the phage are shown in the right panel. A consensus sequence is highlighted. An asterisk indicates the modified cysteine. For the alignment, ESPript program (http://espript.ibcp.fr/)[27] was used. A negative control experiment (blank) was also performed in the absence of T7 phage.

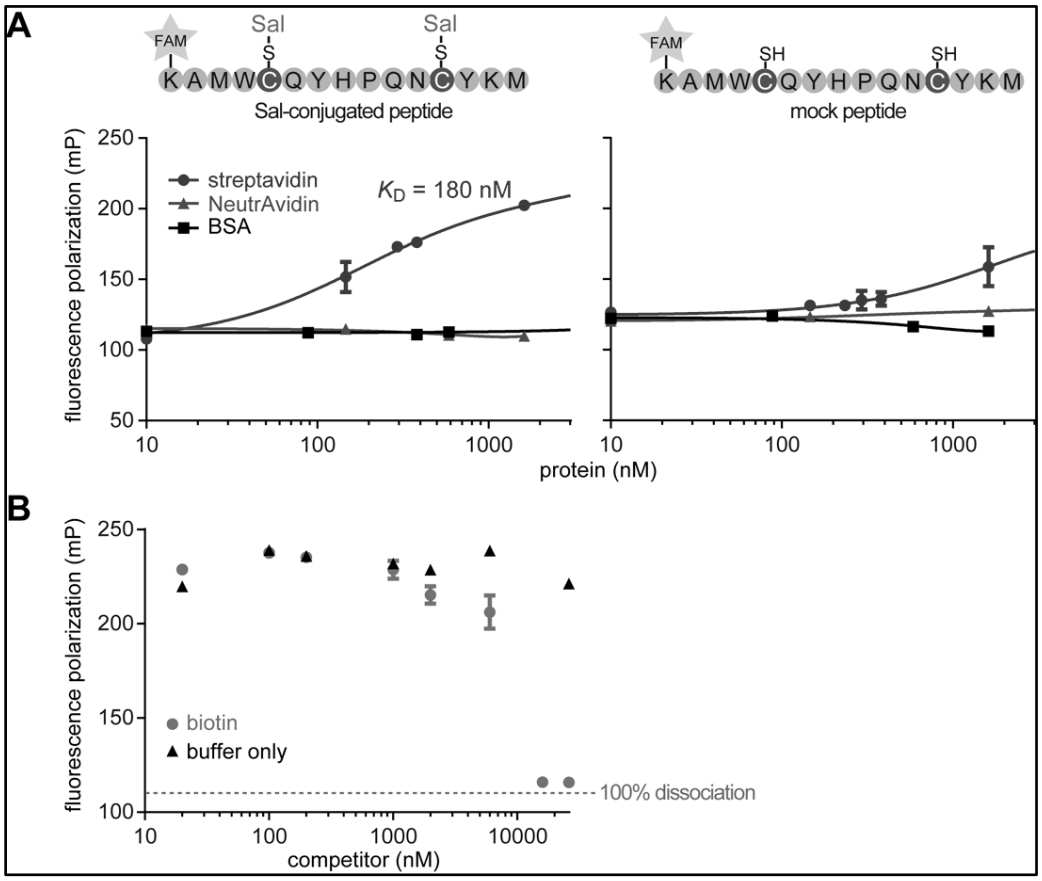


Figure 6. (**A**) Determination of streptavidin-binding affinities of the Sal-conjugated peptide (left panel) and the mock peptide (right panel) by fluorescence polarization assay. The amino acid sequence of the peptide is shown in the upper part. The plots indicate the polarization (mP) of the fluorophore (FAM)-coupled peptides in the presence of various concentrations of target proteins. NeutrAvidin and bovine serum albumin (BSA) were used as mock target proteins. (**B**) Competitive binding assay. Biotin as a competitor was mixed with the Sal-conjugated peptide-streptavidin complex. Error bars represent standard deviations.

■ 3.3 Additional figures and experimental procedures

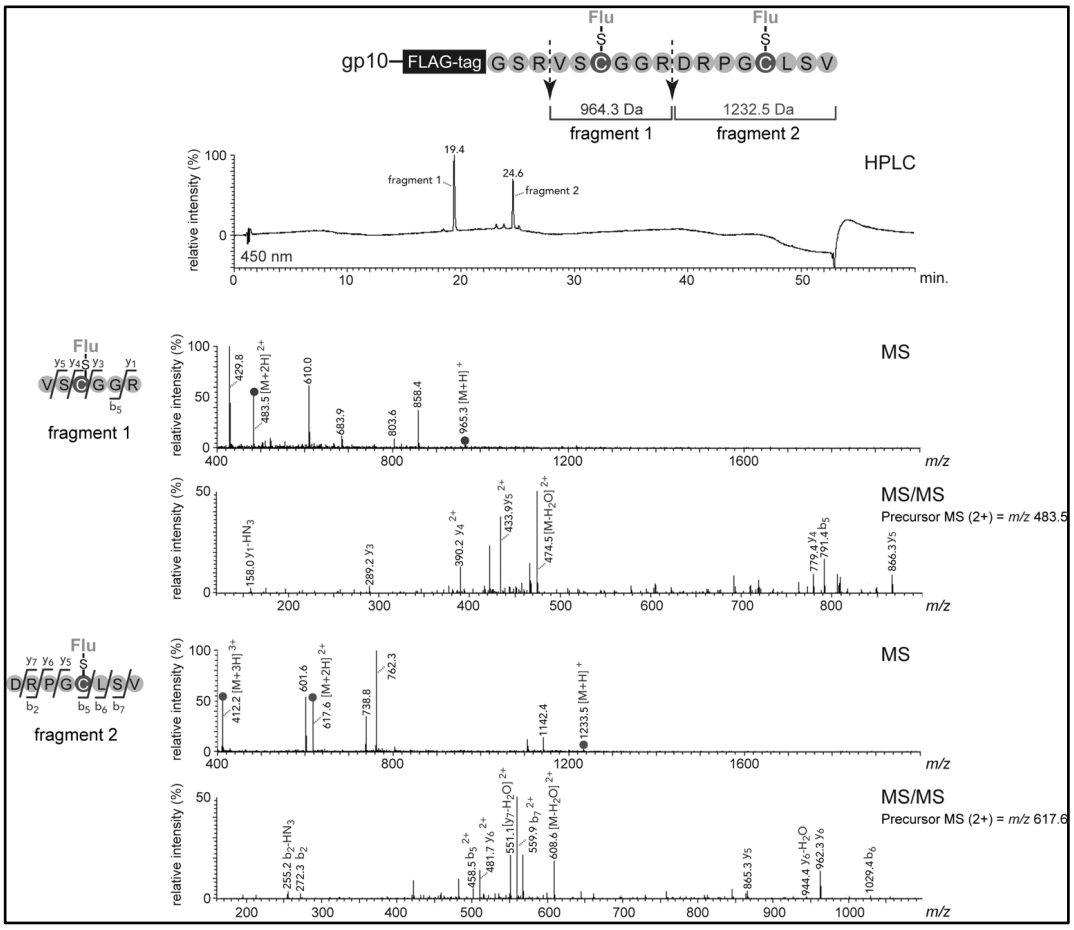


Figure S1. Identification of peptide fragments derived from fluorescein (Flu)-conjugated T7 phage-displayed peptide by LC-MS/MS. Upper panel: 450 nm (Flu absorption under low pH condition) chromatogram of the trypsinized peptide-fused gp10. Note that trypsin could not cleavage before proline. Middle and lower panels: MS and MS/MS spectra correspond to the trypsinized two peptide fragments possessing Flu moiety.

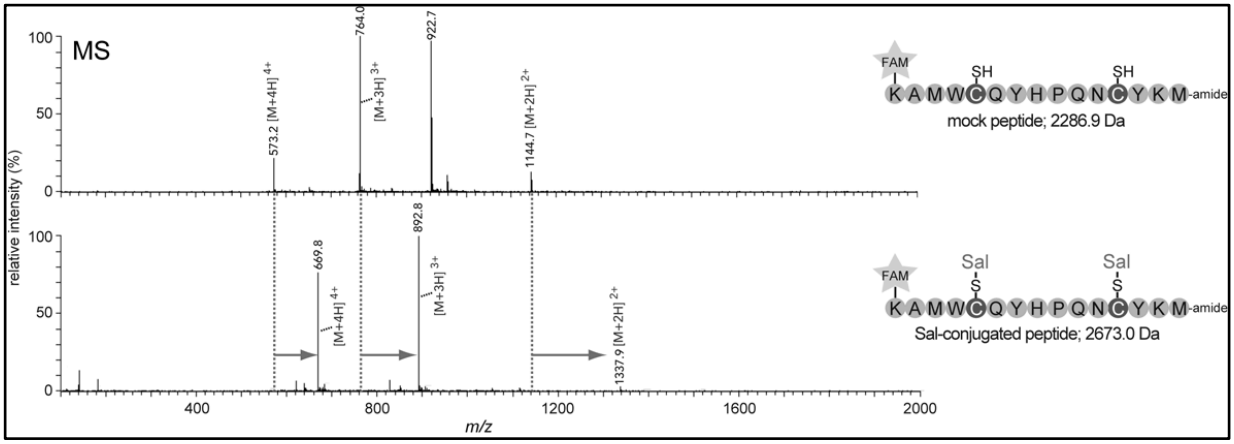


Figure S2. Mass spectrometric analysis of chemically synthesized peptides. MS spectra of mock peptide (upper panel) and Sal-conjugated peptide (lower panel).

General

All experiments were performed with commercially available reagents and kits. Note that no special materials and skills are needed. Contrary to popular belief and the T7Select system manual (Merck Millipore), CsCl step gradient and ultracentrifugation are not necessary to perform all of the experiments to obtain a target-specific binder. For purification of T7 phage at every step by polyethyleneglycol / NaCl precipitation, I only used a conventional centrifugation system which can rotate at 13,000 rpm. In all phage display experiments, Escherichia coli BLT5403 cells were used. Note that medium-high-copied peptide display (200 peptides per virion) is produced by an amplification of T7 phage carrying a T7Select415-1b high-copy vector by using BLT5403 cells.[13, 18]

Construction of T7 phage display libraries

A T7 phage display peptide library (-S-G-G-G-X_3-C-X_6-C-X_3; X represents any amino acid) was independently constructed in the same manner of my method.[10, 11, 13]

Chemical modification of T7 phage-displayed peptide via the 10BASE$_d$-T

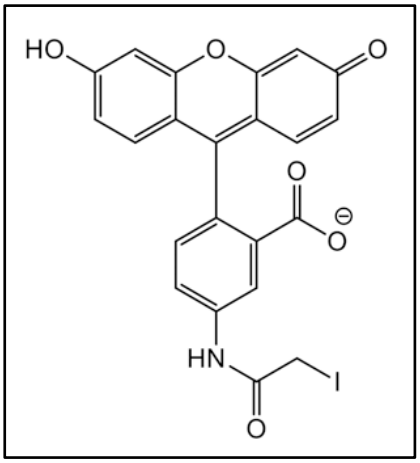
Procedures of standard reaction conditions of the 10BASE$_d$-T are the following.

Tris(2-carboxyethyl)phosphine (TCEP) and 4-iodoacetamidosalicylic acid (Sal-IA; MP Biomedicals, cat. No. 102065) stock aqueous solutions of pH 7 should be prepared by neutralization with NaOHaq in advance. All reactions were carried out in 1.5 mL microcentrifuge tubes. T7 phage particles (approximately 1.0×10^{11} PFU) were resuspended in 700 μL of phosphate-buffered saline (PBS; Nacalai tesque, cat. No. 14249-95) supplemented with 400 mM NaCl, and well dissolved by vortex. At this step, the solution contained 1.0×10^{12} (T7 phage made by T7Select10-3b system for the selection) or 2.0×10^{13} (by T7Select415-1b system for the optimization of Sal-IA concentration) molecules of the T7-displayed peptides. After centrifugation at 12,000 rpm for 5 minutes at room temperature, the supernatant was mixed with neutralized TCEP-NaOH (final concentration of 500 μM) at 4 °C.

For the selection against streptavidin: Neutralized Sal-IA-NaOH was added to the above TCEP-treated phage at a final concentration of 800 μM, and the mixture was incubated at 4 °C for 3 hours in the dark with shaking. To inactivate the unreacted Sal-IA, 2-mercaptoethanol was added to the mixture at a final concentration of 5 mM, and further incubated at 4 °C for several minutes. The T7 phage particles were precipitated with a mixture of polyethylene glycol 6000 (Nacalai tesque) and sodium chloride to final concentrations of 5% (w/v) and 0.5 M, respectively. After centrifugation, the precipitate was dissolved in an appreciate buffer.

For the optimization of Sal-IA concentration: Neutralized Sal-IA-NaOH was added to the above TCEP-treated phage at various concentrations, and the mixture was incubated at 4 °C for 3 hours in the dark with shaking. After the reaction, 5-iodoacetamide-fluorescein (FL-IA) stock solution in dimethyl sulfoxide/water (1:1 by volume) was added at a final concentration of 200 μM, and the mixture was further incubated at 4 °C for 3 hours. FL-IA was purchased from Sigma-Aldrich (cat. No. I9271). The latter reaction with fluorescent FL-IA reacts with all the unreacted SH-groups on the displaying peptide after the conjugation with Sal-IA. Thus, I can

indirectly estimate conversion yield of the Sal-IA treatment by SDS-PAGE followed by densitometric fluorescence analysis of the peptide-fused gp10. Site-specificity of the $10BASE_d$-T by using FL-IA was confirmed by LC-MS/MS (Fig. S1). For the optimization, a model T7 phage with medium-high-copied peptides possessing the -G-S-R-V-S-C-G-G-R-D-R-P-G-C-L-S-V sequence at the C-terminal region of gp10 was used.[13]



Chemical structure of 5-iodoacetamide-fluorescein

In-gel fluorescence imaging

T7 phage particles were dissolved in 1 × sample buffer (62.5 mM tris(hydroxymethyl)aminomethane-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.002% bromophenol blue). The solution was incubated at 95 °C for 5 min, and then subjected to SDS-PAGE. Proteins were resolved by a 10% polyacrylamide gel. After electrophoresis, the FL-conjugated proteins were visualized by in-gel fluorescence imaging using a conventional gel imager (ChemiDoc XRS+, Bio-Rad) excited with UV light.

Mass spectrometric analysis

For mass spectrometric analysis, a PAGE gel was stained with Rapid Stain CBB kit (Nacalai tesque, cat No. 30035-14). The stained protein bands were excised from the

gel. Proteins in the gel were reduced with 25 mM dithiothreitol at 65 °C for 10 min, and then alkylated with 55 mM iodoacetamide at room temperature for 60 min in the dark. Digestion was carried out with modified trypsin (Promega, Madison, WI) at 37 °C overnight. The resulting peptides were analyzed using Agilent 1100 semi-micro HPLC system (Agilent Technologies) equipped with a C_{18} reverse-phase column (Hypersil GOLD, 2.1 × 100 mm, Thermo Scientific) connected to LCQ-Fleet mass spectrometer. The peptides were separated using a 0-50% gradient of acetonitrile containing 0.1% formic acid during 40 min at a flow rate of 300 μL per minute, and then eluted peptides were directly sprayed into the mass spectrometer. The mass spectrometer was operated in the data-dependent mode and externally calibrated. Survey MS scans were acquired in the 300-2000 or 400-2000 m/z ranges. Multiply charged ions of high intensity per scan were fragmented with collision-induced dissociation in the ion trap. A dynamic exclusion window was applied within 30 seconds. All tandem mass spectra were collected using normalized collision energy of 40%. Data were acquired and analyzed with Xcalibur software v. 2.07 (Thermo Scientific).

Biopanning against streptavidin

For biopanning, approximately 8.4×10^{10} PFU of a T7Select10 library (-S-G-G-G-X_3-C-X_6-C-X_3; X represents any randomized amino acid) were modified via the 10BASE$_d$-T. The modified T7 library was dissolved in a selection buffer (PBS supplemented with 0.5% Triton X-100 and 1 mM TCEP-NaOH), and incubated with streptavidin-immobilized nanomagnetic beads (FG streptavidin beads, Tamagawa Seiki, cat No. TAS8848N1170) at 4 °C. The beads were washed three times with 200 μL of the selection buffer, and the streptavidin-bound phages were directly infected and amplified with *E. coli* BLT5403 cells. Stringent conditions were applied to each round by shortening the binding time and by increasing the washing frequency. For binding and washing, an automated bioscreening machine for phage display system (TargetAngler 8, Tamagawa Seiki) was used. After 4 rounds of biopanning, randomly chosen T7 phage clones were subjected to DNA sequencing.

Enzyme-linked immunosorbent assay (ELISA)

830 pmol of streptavidin (Wako Pure Chemical Industries) was dissolved in PBS, and immobilized on each well of a 96-well immunoplate (Nunc MaxiSorp, Thermo Scientific). After washing with PBS, surface of the wells were coated with 1% (w/v) BSA in PBS supplemented with 0.05% Tween-20 at 4°C overnight. Approximately 2.0 × 10^{10} PFU of the T7 phage was suspended in a wash buffer (tris-buffered saline supplemented with 0.5% Triton X-100), and applied to the well plate. The plate was incubated for 1 hour at 25 °C with shaking by using a maximizer (MBR-022UP, Taitec, Japan), and then washed three times with the same buffer. The bound phage was incubated with T7 tail fiber monoclonal antibody (1:5,000 dilution; Merck Millipore, cat No. 71530-3) and anti-mouse IgG HRP-linked antibody (1:5,000 dilution; Cell Signaling, cat No. 7076) for 1 hour at 25 °C with shaking. After washing, o-phenylenediamine dihydrochloride substrate (SigmaFast OPD; Sigma Aldrich, cat No. P9187) was added, and the absorbance was quantified using a microplate reader equipped with a 450 nm band-pass filter (Bio-Rad).

Peptide synthesis

An unmodified fluorescent peptide (H_2N-K-5/6-FAM-A-M-W-C-Q-Y-H-P-Q-N-C-Y-K-M-NH_2) was prepared by solid phase peptide synthesis using a semi-automated personal synthesizer (PetiSyzer®; HiPep Laboratories, Japan) as described previously.[28] Fmoc amino acids and Fmoc-Lys(5/6-FAM) were purchased from HiPep Laboratories and AAT Bioquest, respectively. The solid phase synthesis was performed on Fmoc-NH-SAL-PEG resin (Watanabe Chemical Industries, LTD., cat No. A00213). Chain elongation was carried out by using 1-Hydroxy-7-azabenzotriazole (HOAt) and N,N,N',N'-tetramethyl-O-(7-azabenzo-triazol-1-yl)uronium hexafluorophosphate (HATU) as the coupling reagent in the presence of diisopropylethylamine (DIPEA) / N,N-dimethylformamide (DMF) / N-Methylpryrrolidone (NMP). The coupling was set

to 50 °C for 40 minutes. The resin-bound peptide was cleaved by trifluoroacetic acid (TFA) / water / triisopropylsilane (TIS) / 1,2-ethandithiol (EDT) (94/ 2.5/ 1/ 2.5 (v/v/v/v)) for 1 hour at 50 °C. The crude peptide was dissolved in 0.1% formic acid (aq) and then purified with reverse-phase HPLC (Shimadzu, Japan) equipped with XTerra Prep MS C_{18} column (10 × 50 mm, Waters) and XBridge Prep C_{18} column (10 × 50 mm, Waters). The peptide was separated using a 0-100% linear gradient of acetonitrile containing 0.1% formic acid during 12 min at a flow rate of 4 mL per minute. Characterization of the peptides was performed by LC-MS/MS (Fig. S2). Purity was estimated to be above 95%. For the conjugation of Sal, the neutralized alkylating reagent (Sal-IA-NaOH) and neutralized TCEP (pH 7) were mixed with each peptide (100 μM) in a phosphate buffer (20 mM phosphate-KOH, pH 7.4) at final concentrations of 1 mM and 500 μM, respectively. The mixture was incubated overnight at 37 °C in the dark with shaking. After addition of formic acid at a final concentration of 2%, the peptide was purified with reverse-phase HPLC. Purity was estimated to be above 90%.

Fluorescence polarization assay

Fluorescence polarization was measured with a HYBRID-3000ES (Photoscience, Japan) equipped with appropriate filters (Ex. 480 nm and Em. 535/40 nm). The Sal-conjugated fluorescent peptide (4 pmol) was incubated with various concentrations of streptavidin, NeutrAvidin, or bovine serum albumin (BSA) in tris-buffered saline (50 mM Tris-HCl, pH 8.0, 150 mM NaCl) supplemented with 1 mM TCEP-NaOH at 30°C. Concentration of the fluorescent peptide was determined by absorption coefficient at 495 nm. NeutrAvidin® and BSA were purchased from Thermo Scientific and Nacalai tesque, respectively. Concentrations of streptavidin and NeutrAvidin were determined by absorption coefficient at 280 nm. Concentration of BSA was determined by Bradford protein assay (Bio-Rad). Klotz plot was generated by GraphPad Prism software 6.0 (GraphPad Software, San Diego, CA), and the sigmoid curve was fitted with non-linear least squares analysis to obtain the dissociation constant. For the negative control

experiment, the Sal-unmodified fluorescent peptide (4 pmol) was used for the fluorescence polarization assay with the same procedure described above. For the competitive binding assay, various concentrations of biotin were added to the solution containing Sal-conjugated peptide-streptavidin complex (4 pmol of the peptide and 3 nmol of streptavidin; 20 nM and 15 μM, respectively). Biotin was purchased from New England Biolabs.

■ 3.4 References

[1] A. Angelini, C. Heinis, *Curr. Opin. Chem. Biol.,* 2011, **15** (3), 355-61.

[2] S. Ng, M.R. Jafari, R. Derda, *ACS Chem. Biol.,* 2012, **7** (1), 123-38.

[3] L. Jespers, T.P. Bonnert, G. Winter, *Prot. Eng. Des. Sel.,* 2004, **17** (10), 709-13.

[4] C. Heinis, T. Rutherford, S. Freund, G. Winter, *Nat. Chem. Biol.,* 2009, **5** (7), 502-507.

[5] V. Baeriswyl, S. Calzavarini, C. Gerschheimer, P. Diderich, A. Angelillo-Scherrer, C. Heinis, *J. Med. Chem.,* 2013, **56** (9), 3742-3746.

[6] K. Arai, H. Tsutsumi, H. Mihara, *Bioorg. Med. Chem. Lett.,* 2013, **23** (17), 4940-4943.

[7] M. R. Jafari, L. Deng, P.I. Kitov, S. Ng, W.L. Matochko, K.F. Tjhung, A. Zeberoff, A. Elias, J.S. Klassen, R. Derda, *ACS Chem. Biol.,* **2013**, DOI: 10.1021/cb4006722.

[8] B. Santoso, S. Lam, B.W. Murray, G. Chen, *Bioorg. Med. Chem. Lett.,* 2013, **23** (20), 5680-3.

[9] S. Ng, M.R. Jafari, W.L. Matochko, R. Derda, *ACS Chem. Biol.,* 2012, **7** (9), 1482-7.

[10] K. Sakamoto, Y. Ito, T. Hatanaka, P.B. Soni,, T. Mori, K. Sugimura, *J. Biol. Chem.,* 2009, **284** (15), 9986-9993.

[11] T. Hatanaka, S. Ohzono, M. Park, K. Sakamoto, S. Tsukamoto, R. Sugita, H. Ishitobi, T. Mori, O. Ito, K. Sorajo, K. Sugimura, S. Ham, Y. Ito, *J. Biol. Chem.,* 2012, **287** (51), 43126-36.

[12] K. Fukunaga, M. Taki, *J. Nucl. Acids,* 2012, **2012**, 295719.

[13] K. Fukunaga, T. Hatanaka, Y. Ito, M. Taki, *Mol. BioSyst.,* 2013, **9** (12), 2988-2991.

[14] F. Forouhar, Y. Yang, D. Kumar, Y. Chen, E. Fridman, S.W. Park, Y. Chiang, T.B. Acton, G.T. Montelione, E. Pichersky, D.F. Klessig, L. Tong, *Proc. Natl. Acd. Sci. USA,* 2005, **102** (5), 1773-1778.

[15] J. Seidler, S.L. McGovern, T.N. Doman, B.K. Shoichet, *J. Med. Chem.,* 2003, **46** (21), 4477-86.

[16] G.P. SmitH, V.A. Petrenko, *Chem. Rev.,* 1997, **97** (2), 391-410.

[17] A. Rosenberg, K. Griffin, F.W. Studier, M. McCormick, J. Berg, R. Novy, R.

Mierendorf, *inNovations,* 1998, **6**, 1-6.

[18] K.D. Wittrup, G.L. Verdine, *Methods Enzymol.,* 2012, **503**, xiii-xiv.

[19] S. Chen, J. Touati, C. Heinis, *Chem. Commun. (Camb),* 2014, DOI: 10.1039/c3cc47496h

[20] Z.M. Carrico, M.E. Farkas, Y. Zhou, S.C. Hsiao, J.D. Marks, H. Chokhawala, D.S. Clark, M.B. Francis, *ACS Nano,* 2012, **6** (8), 6675-80.

[21] M. Vodnik, U. Zager, B. Strukelj, M. Lunder, *Molecules,* 2011, **16** (1), 790-817.

[22] L.R. Krumpe, A.J. Atkinson, G.W. Smythers, A. Kandel, K. Schumacher, J.B. McMahon, L. Makowski, T. Mori, *Proteomics,* 2006, **6** (15), 4210-22.

[23] B.K. Kay, N.B. Adey, Y.S. He, J.P. Manfredi, A.H. Mataragnon, D,M, Fowlkes, *Gene,* 1993, **128** (1), 59-65.

[24] J.R. Frost, J.M. Smith, R. Fasan, *Curr. Opin. Struct. Biol.,* 2013, **23** (4), 571-80.

[25] B.A. Katz, R.T. Cass, *J. Biol. Chem.,* 1997, **272** (20), 13220-8.

[26] A.D. Keefe, D.S. Wilson, B. Seelig, J.W. Szostak, *Prot. Expr. Purif.,* 2001, **23** (3), 440-6.

[27] P. Gouet, X. Robert, E. Courcelle, *Nucl. Acids Res.,* 2003, **31** (13), 3320-3.

[28] T. Hamamoto, M. Sisido, T. Ohtsuki, M. Taki, *Chem. Commun. (Camb),* 2011, **47** (32), 9116-8.

[29] S. Li, R.W. Roberts, *Chem. Biol.,* 2003, **10** (3), 233-9.

[30] D.G. Lloyd, C.L. Buenemann, N.P. Todorov, D.T. Manallack, P.M. Dean, *J. Med. Chem.,* 2004, **47** (3), 493-6.

[31] S. Reardon, *Nature,* 2013, **503**, 449-50.

[32] S.Y. Yang, *Drug Discov. Today,* 2010, **15** (11-12), 444-50.

CHAPTER 4

Construction of a crown ether-like macrocyclic library and selection of Hsp90-binders

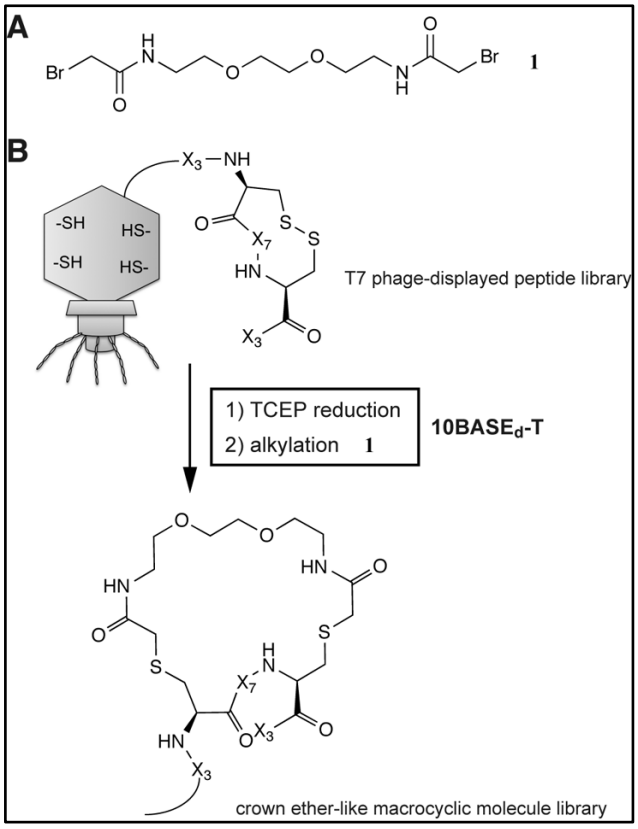
■ Abstract

By using the 10BASE$_d$-T, crown ether-like macrocyclic library possessing randomized peptide is synthesized on bacteriophage T7. Among 1.5×10^9 diversity of the supramolecules, the Hsp90 N-terminal domain-specific binder is discovered.

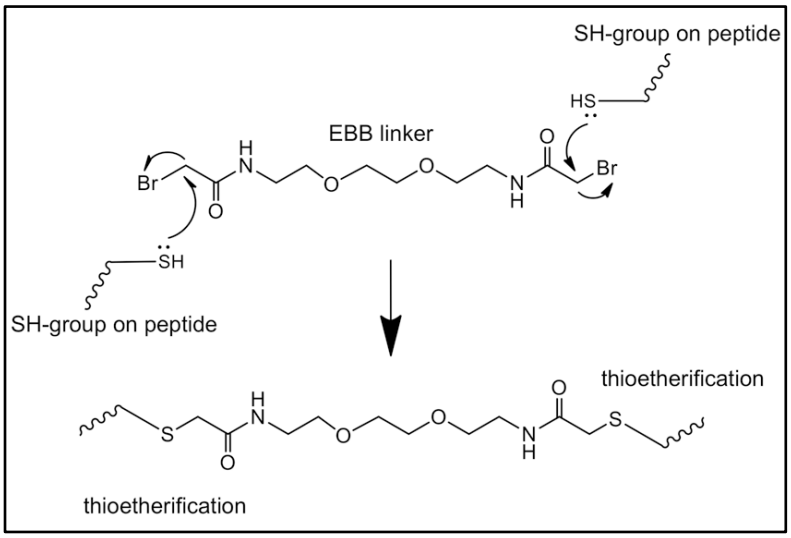
■ 4.1 Introduction

Macrocyclic artificial supramolecules (*e.g.,* cyclophanes,[1] rotaxanes,[2] calixarenes,[3-5] porphirins,[6,7] and fullerene adducts[8]) have been attracting attentions for broad applications such as biomedical, electrochemical, and photophysical materials. Among them, crown ethers and these analogues[9, 10] possess biocompatibility because hydrophilic oligoethylene structure increases water solubility, hence prevents aggregation. The ether oxygen atoms would form hydrogen bondings with basic amino acids of protein.[11] Despite the potential usefulness of the crown analogues, comprehensive study for biological application, such as discovery of target protein-specific binders, has never been reported. It is most plausibly because that structural diversities of the rationally designed analogues are too small to find strong binders toward the complex biomolecules. To increase diversity of the crown analogues, combinatorial synthetic approach is often used.[12] Nevertheless, the size of the library is far enough.

Recently, I have established a library construction system by conjugation between artificial molecules and randomized library peptides; the site-specific conjugation at designated cysteines in the randomized peptide region on a capsid protein (gp10) of T7 phage has been achieved.[13] This gp*10 based-t*hioetherificaion (10BASE$_d$-T) is carried out in one-pot without side reactions or loss of phage infectivity. By using the 10BASE$_d$-T, here I cyclized both ends of oligoethyleneglycol unit by randomized peptide via the thioether linkage, to afford the crown analogue library with vast diversity (i.e., 10^9).



Scheme 1. (**A**) Chemical structure of EBB: 1. (**B**) Construction of a crown ether-like macrocyclic library through the 10BASE$_d$-T. X represents randomized amino acid.



Scheme 2. Thioetherification of SH-groups on peptide by S_N2 reaction.

■ 4.2 Results and discussion

I synthesized a cysteine-reactive bifunctional synthon, *N,N'*-[1,2-ethanediyl-oxy-2,1-ethanediyl]bis(2-bromoacetamide) (EBB; Scheme 1A) as the supramolecule core, and the 10BASE$_d$-T was performed as follows (Scheme 1B) : T7 phage-displayed peptides (1.0×10^{11} PFU) were mixed with EBB in 700 μL of phosphate-buffered saline (pH 7.4) supplemented with 500 μM tris(2-carboxyethyl)phosphine (TCEP) and 400 mM NaCl. After 3 hours reaction at 4 °C, 2-mercaptoethanol (5 mM) was added to quench unreacted EBB, and the mixture was incubated for 5 min at 4 °C. To confirm the cyclization of the synthon with T7 phage-displayed peptide linker, I used a model phage displaying linker peptides (-G-S-R-V-S-C-G-G-R-D-R-P-G-C-L-S-V).[13] After the 10BASE$_d$-T against the model T7 phage, total proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by coomassie brilliant blue staining. The linker-fused T7 capsid protein (gp10) was excised from the gel, and then digested with lysyl endopeptidase. The resulting peptide fragments were analyzed by LC-MS/MS. MS and MS/MS results indicate that Cys in the model T7 phage-displayed peptide linker were site-specifically conjugated with EBB (Fig. S1). Generally, acquisition of a MS/MS spectrum of a ring-forming peptide region is difficult.[14, 15] However, I tried in-depth mass spectrometric analysis of the crown analogue on T7 phage, to clarify amino acid sequence of the ring-forming peptide region. Here I performed a LC-MS/MS analysis of the macrocycle by enzymatic digestion of the ring-forming peptide (Fig. 1A). After cleavage of the ring by trypsinization, the resultant branched peptide fused to both ends of EBB was analyzed by LC-MS/MS. Two classes of fragment ions were detected in the MS/MS analysis, and consistent with amino acid sequence inside the ring (Fig. 1B). Consequently, the model crown analogue on T7 phage was unambiguously identified.

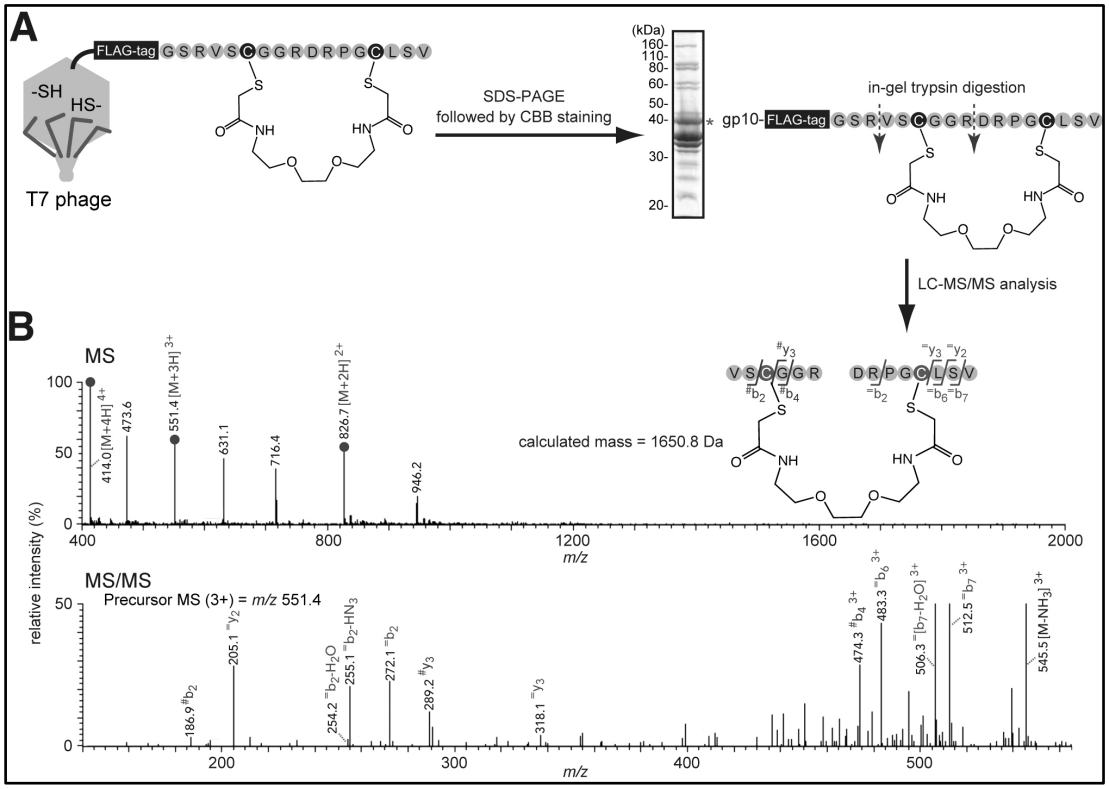


Figure 2. (**A**) Strategy for identification of a model crown analogue displayed on T7 phage. A T7 phage-displaying model linker peptide was conjugated with EBB via the $10BASE_d$-T, and then separated into a subunit by SDS-PAGE. After coomassie brilliant blue (CBB) staining, the asterisk corresponding to the crown-fused gp10 was excised and subjected to in-gel trypsinization followed by LC-MS/MS analysis. Arrows indicate trypsin cleavage site. (**B**) MS analysis of the ring-opening crown analogue. Above panel: MS spectrum. A series of multiple charged ions (circles) were detected, and consistent with theoretical *m/z* values of the crown analogue. Other ions were considered as trypsinized gp10 fragments (*m/z* 946.2, 631.1 and 473.6: D-Q-A-A-Y-L-A-P-G-E-N-L-D-D-K-R-K, *m/z* 716.4: D-L-A-L-E-R). Lower panel: MS/MS spectrum. Trypsin could not cleave arginine C-terminus before proline as reported previously.[8]

As a target of biopanning using the crown analogue library, I used heat shock protein 90 (Hsp90), which plays a crucial role in protein homeostasis, cell signaling, and stress response.[16-18] Hsp90 is a highly conserved molecular chaperone which governs cellular protein quality control,[19, 20] and considered as an important class of drug target in cancer therapy.[21, 22] Prior to biopanning, EBB was cyclized with T7

phage-displayed peptide linker library (-S-G-G-G-X_3-C-X_7-C-X_3; where X represents any amino acids)[13, 23] via the 10BASE$_d$-T as shown in Scheme 1B. Next, I examined the infectivity of the modified T7 phage library by plaque assay, and the infectivity titer was fully retained (Fig. S2).

Six rounds of biopanning were performed against biotinlylated-Hsp90, and enrichment of Hsp90 binders was assessed by enzyme-linked immunosorbent assay (ELISA). After the 6 rounds of biopanning, the crown analogues on T7 phage polyclones showed the strongest binding to Hsp90, whereas ones lacking the crown core structure did not (Fig. S3A). Among 11 of randomly chosen T7 phage monoclone, 10 clones had the same linker sequences, -R-S-W-C*-R-K-S-R-K-N-S-G-G-G-L-V-W-C*-F (Cys are conjugated with EBB) (Fig. S3B). This was an unexpected result, because the initial library was designed to be displaying -X_3-C-X_7-C-X_3 peptide linkers; DNA sequencing of monoclones randomly chosen from the initial library supported that the peptide linkers were correctly encoded with no bias (data not shown). Thus, I speculate that trace amounts of the crown analogue with the longer peptide linker, which might be encoded by misligated DNA fragment, was enriched by biopanning. To confirm the effect of a crown moiety for the binding, biopanning by using the *naïve* cyclic peptide library with conventional disulfide (S-S) bridge (Scheme 1B, upper) was also performed. By sequence analyses, most abundant sequence -R-M-T-C*-Y-D-K-Q-H-H-H-C*-E-T-W (C* forms intramolecular disulfide bond) was obtained (Fig. S4B). This peptide also bound to Hsp90 NTD (data not shown), however the sequence was completely different from what was discovered from the crown-ether like supramolecule library; only N-terminal arginine and C-terminal hydrophobic aromatic amino acid (tryptophan) were identical. This indicates that the crown moiety strongly affects geometry of the whole macrocycle structure of the naïve library, to generate a novel supramolecular library.

Next, I examined the crown analogue-binding site of Hsp90. Hsp90 is structurally divided into three domains: N-terminal (NTD), middle (MD), and C-terminal (CTD) domains (Fig. 2A).[24] Using each glutathione *S*-transferase (GST)-fused Hsp90

domain,[25] GST-pull down assay was carried out. I found that the crown ether analogue on T7 phage monoclone exclusively bound to the NTD of Hsp90 (Fig. 2A). To determine affinity of the Hsp90 NTD-binding crown analogue, I synthesized the linker peptide with solid-phase peptide synthesis followed by cyclization with EBB core. Isothermal titration calorimetry (ITC) measurement was performed to obtain the dissociation constant (K_D) as well as thermodynamic parameters. The crown analogue bound to GST-Hsp90 NTD with the K_D value of 1.7 ± 0.5 μM, whereas the linker peptide itself almost did not (Fig. 2B). This suggests that both structures of the crown moiety and the rest peptide linker are essential for the Hsp90-specific binding. Also, favorable enthalpy change (ΔH) was observed, suggesting that hydrogen bonding and van der Waals force contributes to the interaction between the crown analogue and Hsp90 NTD. To investigate secondary structure of the macrocycle, circular dichroism (CD) spectroscopy was performed. CD spectrum showed that the crown analogue has a disordered structure (Fig. S5). This structural flexibility of the crown analogue might be important in the interaction, because Hsp90 recognizes unfolded substrates.[19]

Geldanamycin (GA) is an anticancer natural product,[21, 22] which binds to the ATP-binding pocket on the NTD of Hsp90.[24] I here examined whether the crown analogue competes with GA for the Hsp90 NTD-binding. Using a fluorescein-5-isothiocyanate labelled GA (GA-FITC), fluorescence polarization (FP) competition assay was performed. Interaction between GA-FITC and GST-Hsp90 NTD was disrupted in the presence of non-labelled geldanamycin in a concentration dependent manner (Fig. S6B). On the other hand, the interaction was not inhibited in the presence of the crown analogue, suggesting that the macrocycle did not bind to the ATP-binding pocket. Almost all reported Hsp90 inhibitors are ATP competitors.[21] Thus, the crown analogue will possibly be a novel Hsp90 inhibitor with a different inhibition mechanism, such as celastrol, which is an Hsp90 NTD-binding natural terpenoid.[26]

In conclusion, I demonstrated the first example to construct a supramolecular library with vast diversity on T7 phage, and successfully found a target protein-specific binder. Enthalpy change was strongly contributed to the binding between the discovered

crown analogue and the Hsp90 NTD. I envisage that discovery of functional supermolecules will be accelerated by the ensemble of rationally-designed artificial supramolecule cores and genetically-encoded random (poly-)peptide linkers. In parallel with finding the crown-based binders for different target biomolecules, I am now trying to construct a novel artificial libraries of multicyclic structures to find supramolecules with greater binding affinity.

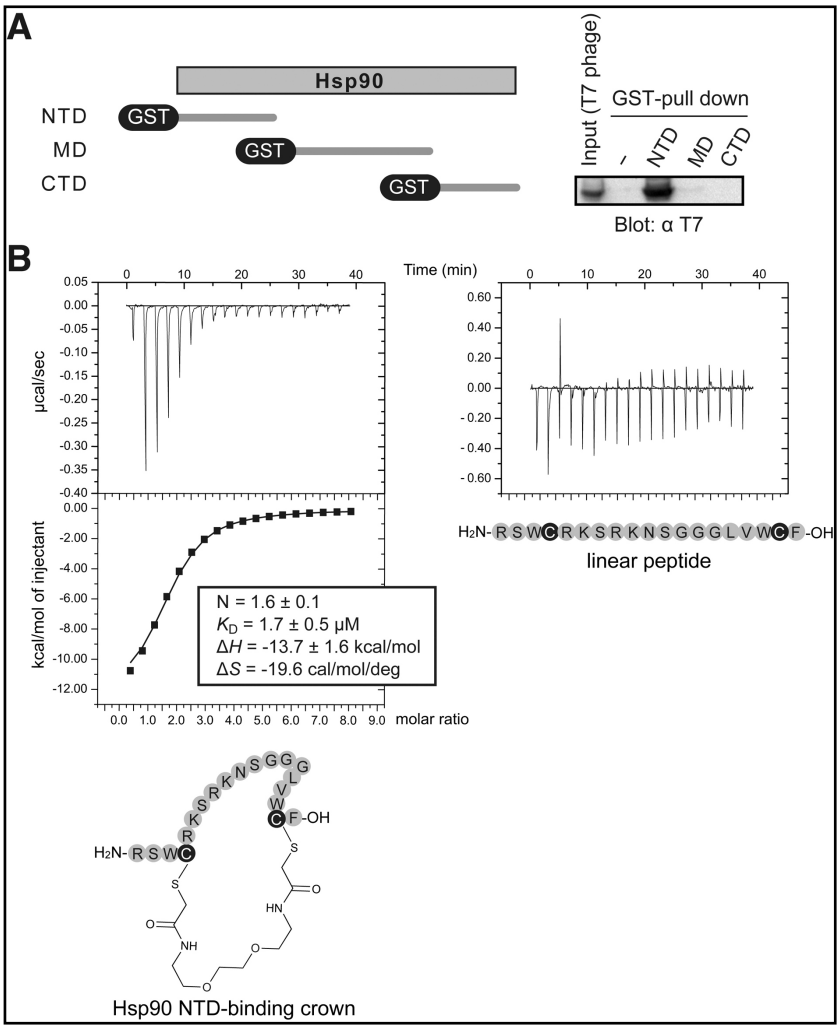


Figure 2. (**A**) GST pull-down assay. EBB-modified T7 phage monoclone (-R-S-W-C*-R-K-S-R-K-N-S-G-G-G-L-V-W-C*-F; *Cys were conjugated with EBB core) was used as the input. N-terminal (NTD), middle (MD), and C-terminal (CTD) domains of Hsp90 are schematically shown on the left. (**B**) Isothermal titration calorimetry profiles of titrations of GST-Hsp90 NTD with crown analogue (left) and linear linker possessing the same peptide sequence (right). N: number of binding sites, K_D: dissociation constant, ΔH: enthalpy change, ΔS: entropy change.

■ 4.3 Additional figures and experimental procedures

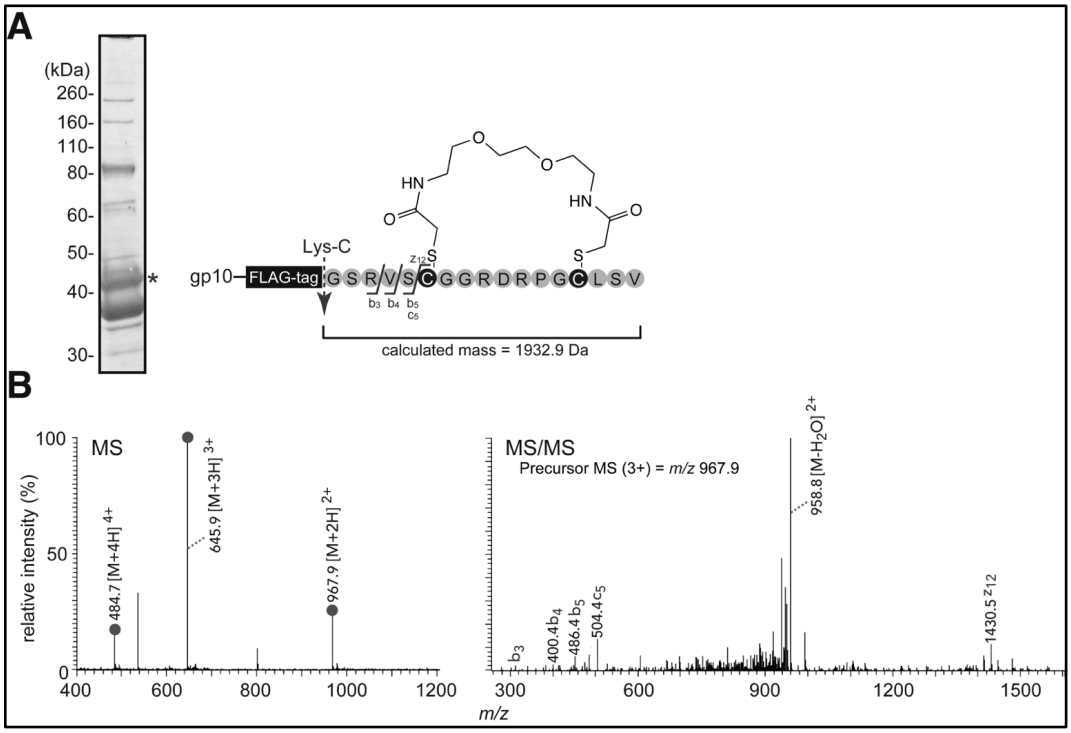


Figure S1. Tandem mass spectrometric analysis of the crown analogue displayed on T7 phage. (**A**) A model T7 phage monoclone modified by 10BASE$_d$-T was separated into subunits by SDS-PAGE. After coomassie brilliant blue staining (left panel), the protein band corresponding to the crown molecule-fused gp10 (asterisk) was excised and subjected to in-gel lysyl endopeptidase (Lys-C) digestion followed by LC-MS/MS analysis. A blue arrow indicates the Lys-C cleavage site. (**B**) MS spectrum (left panel). A series of multiple charged ions (circles) were detected, and consistent with calculated *m/z* values of the crown molecule. MS/MS spectrum (right panel).

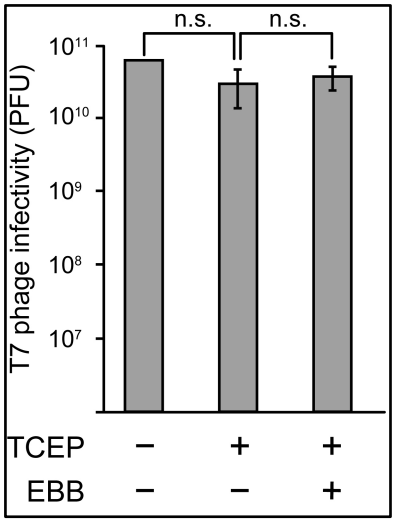


Figure S2. Infectivity of modified T7 phage. A T7 phage library (-S-G-G-G-X_3-C-X_7-C-X_3; X represents any randomized amino acid) was treated with or without EBB (500 μM) in the presence of TCEP (500 μM) under the standard conditions. The number of plaque forming units was determined by a serial dilution method and plaque assay. The graph summarizes the results of three independent experiments. Error bars represent standard deviations. Statistical analysis was performed by unpaired Student's *t*- test. n.s., not significant.

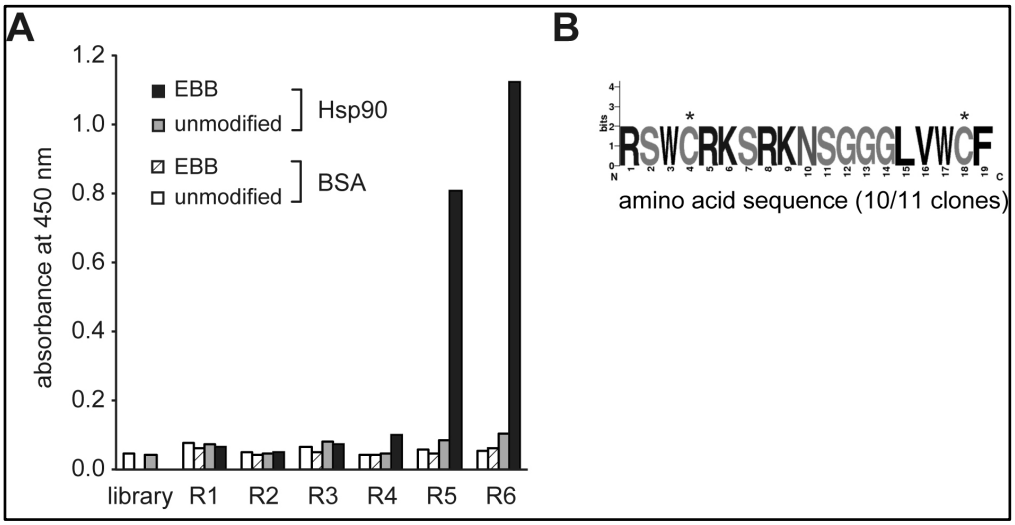


Figure S3. Biopanning against Hsp90 using the crown ether-like supramolecular library (-S-G-G-G-X_3-C^*-X_7-C^*-X_3; Cys are conjugated with EBB). (**A**) T7 phage polyclones after each round (R) of the biopanning were modified with EBB. Both the modified and unmodified T7 phage polyclones were subjected to ELISA. Bovine serum albumin (BSA) served as a mock protein. (**B**) T7 phage monoclones were chosen from phage pool after the 6 rounds of the

biopanning, and then subjected to DNA sequencing. Sequence logo was generated by WebLogo program.

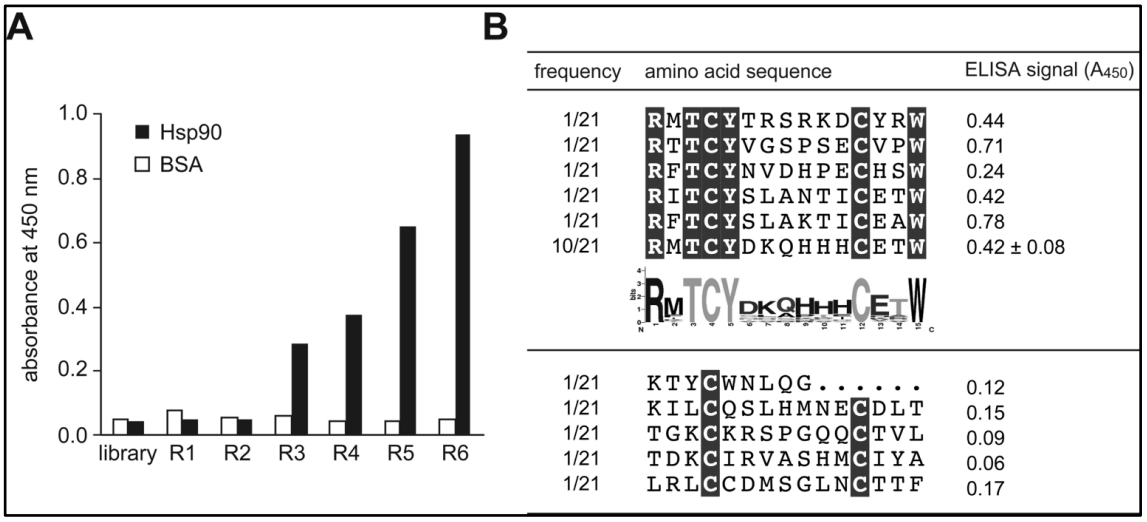


Figure S4. Biopanning against Hsp90 using the *naïve* T7 phage library (-S-G-G-G-X_3-C-X_7-C-X_3). (**A**) ELISA result shows enrichment of Hsp90-binding phage through 6 rounds of the biopanning. (**B**) Sequence alignment of Hsp90 binder (upper column) and non-binder (lower column). The alignment was generated by ESPript program (http://espript.ibcp.fr). Consensus sequences are highlighted. Frequency of each amino acid sequences are shown on the left side, and summarized into a sequence logo by WebLogo program. Hsp90-binding avidities are shown on right side. The consensus sequence was completely different from what was discovered in the Hsp90 NTD-binding crown molecule (Fig. S3B); only N-terminal arginine and C-terminal hydrophobic aromatic amino acid (tryptophan) were identical.

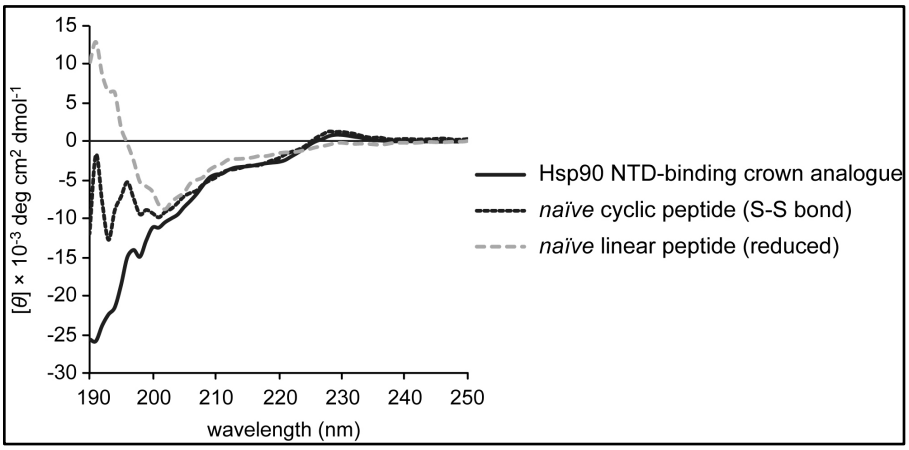


Figure S5. CD spectra of Hsp90 NTD-binding crown molecule and synthetic peptide linkers.

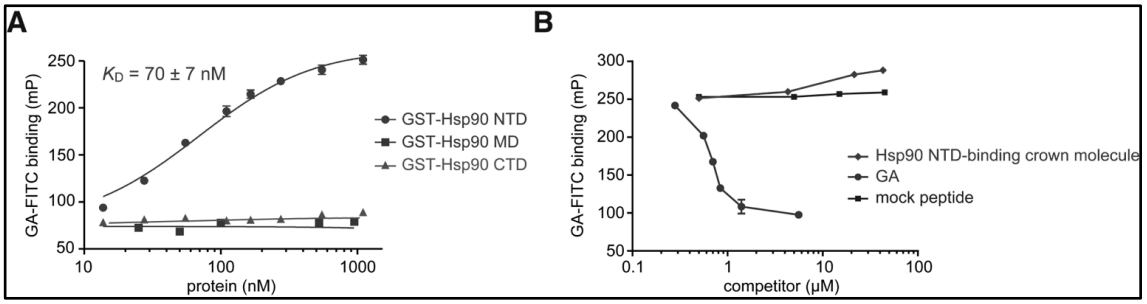
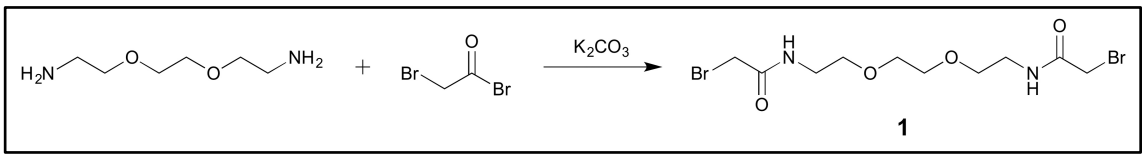


Figure S6. (**A**) Determination of Hsp90 N-terminal domain (NTD)-binding affinity of fluorophore-conjugated geldanamycin (GA) by FP assay. Middle domain (MD) and C-terminal domain (CTD) of Hsp90 were used as mock proteins for negative controls. Error bars represent standard deviations. The binding affinity of geldanamycin to Hsp90 NTD was close to a previously reported one.[3] (**B**) Competitive binding assay. Non-labeled GA and mock peptide (GCDPETGTCG) served as a positive and negative control, respectively. The Hsp90 NTD-binding crown molecule did not compete with GA-FITC, or possibly might enhance binding of GA-FITC to Hsp90.

Synthesis of EBB



N,N'-[1,2-ethanediyl-oxy-2,1-ethanediyl]bis(2-bromoacetamide) (EBB; **1**) was prepared as previously reported[4] with minor modifications. Briefly, 2,2'-(ethylenedioxy)bis(ethylamine) (25 mmol; cat. No. 385506, Sigma-Aldrich) and pottasium carbonate (60 mmol) were mixed in 100 mL of H_2O/AcOEt = 1:1 solution. Then, bromoacetyl bromide (75 mmol; cat. No. B56412, Sigma-Aldrich) was added, and the mixture was stirred for 4 hours at room temperature. The organic layer was collected, and then the solvent was evaporated (860 mg: 81% yield). The crude reaction product (370 mg) was dissolved in pure water, and purified by reverse-phase middle pressure liquid chromatography (Yamazen ODS column 26 × 300 mm, flow rate 20 mL/min with gradient 5-100% MeOH in pure water over 20 min). The fractionated sample was lyophilized (55% yield). ESI-IT-MS and ^{1}H NMR (300 MHz, $CDCl_3$) spectrum were shown in below (Fig. S7).

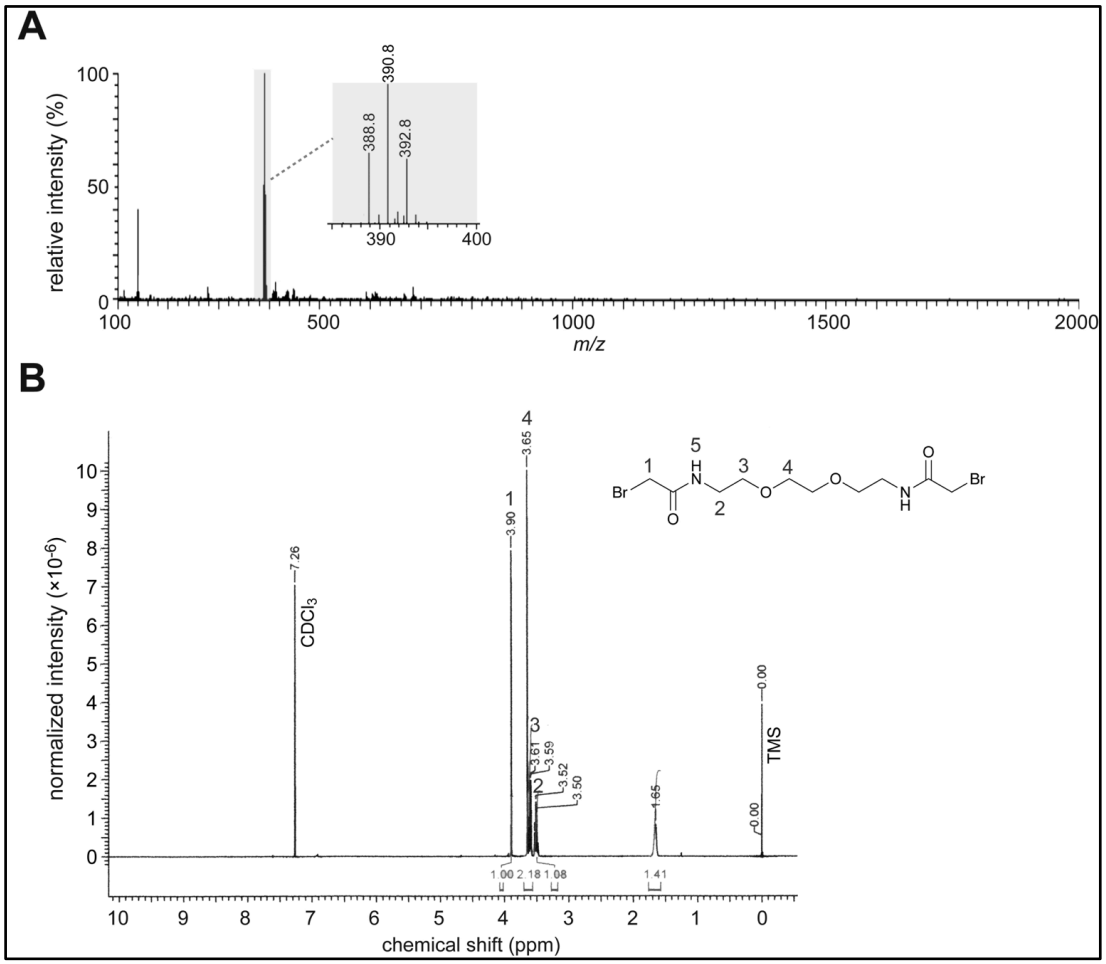


Figure S7. (**A**) MS spectrum (observed *m/z* values = 388.8, 390.8 and 392.8; calculated *m/z* values = 389.0, 341.0 and 393.0) and (**B**) ^{1}H NMR spectrum of EBB.

Construction of a crown ether-like supramolecular library via the 10BASE$_d$-T

Synthesis of the crown analogue on T7 phage was performed as described previously.[5] Standard reaction condition of gp10 based-thioetherification (10BASE$_d$-T) is the following: T7 phage particles (approximately 1.0×10^{11} PFU) were well suspended by sonication or vortex in a 700 μL of phosphate buffered saline (PBS) supplemented with 400 mM NaCl. After centrifugation at 12,000 rpm for 5 minutes at room temperature, the supernatant was mixed with neutralized TCEP aqueous solution at a final concentration of 500 μM at 4 °C: optimal molar concentration of EBB was estimated at 0.5 to 1.0 mM by LC-MS-based quantification of an intact peptide. Conversion yield to a crown analogue was maximum 80% (data not shown). EBB aqueous solution was added at a final concentration of 500 μM, and the mixture was

incubated at 4 °C for 3 hours in the dark. To inactivate the unreacted EBB, 2-mercaptoethanol was added to the mixture at a final concentration of 5 mM, and further incubated at 4 °C for several minutes. The T7 phage particles were precipitated with a mixture of polyethylene glycol 6000 and sodium chloride to final concentrations of 5% (w/v) and 0.5 M, respectively. After centrifugation at 15,000 rpm for 10 minutes at 4 °C, the precipitate was suspended in an appreciate buffer.

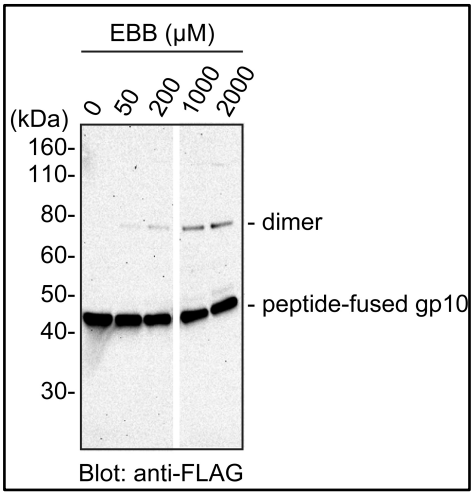


Figure S8. Western blotting against peptide-fused gp10. Trace amount of gp10 dimer was formed in the EBB-concentration dependent manner.

Mass spectrometric analysis

Mass spectrometric analysis was performed as described previously. The gel was stained with rapid stain CBB kit (Nacalai, Japan), and then the stained protein band was excised from the gel. The protein samples were reduced with 25 mM DTT at 55 °C for 30 min, and then alkylated with 55 mM iodoacetamide at room temperature for 30 min in the dark. Digestion was carried out with modified trypsin (Trypsin Gold, Promega) or lysyl endopeptidase (Wako, Japan) at 37 °C overnight. The resulting peptides were analyzed using an Agilent 1100 HPLC system (Agilent Technologies) equipped with a C18 reverse-phase column (Hypersil GOLD, 2.1 × 100 mm, Thermo Fisher Scientific) connected to a LCQ-Fleet ion trap mass spectrometer. The peptides were separated

using a 0-50% gradient of acetonitrile containing 0.1% formic acid during 40 min at a flow rate of 300 μL per a minute, and then eluted peptides were directly sprayed into the mass spectrometer. The mass spectrometer was operated in the data-dependent mode and externally calibrated. Survey MS scans were acquired in the 100-2000 or 400-2000 *m*/*z* range. Multiply charged ions of high intensity per scan were fragmented with CID in the ion trap. A dynamic exclusion window was applied within 30 seconds. All tandem mass spectra were collected using normalized collision energy of 40%. Data were acquired and analyzed with Xcalibur software v. 2.07 (Thermo Fisher).

Biotinylation of Hsp90

Porcine Hsp90 (a gift from Dr. Yasufumi Minami, Maebashi Institute of Technology, Japan) was biotinylated and purified with a kit (Biotin Labeling Kit –NH_2, DOJINDO, Japan) according to the manufacturer's instruction. The biotinylation of Hsp90 was confirmed by Western blotting (Fig. S9A). From densitometric quantification, it was estimated that approximately 9 molecules of biotin were conjugated to Hsp90 single molecule. Using FP assay (see FP assay section), we confirmed that the ATP-binding pocket of biotinylated-Hsp90 almost remained intact (see below; Fig. S9B).

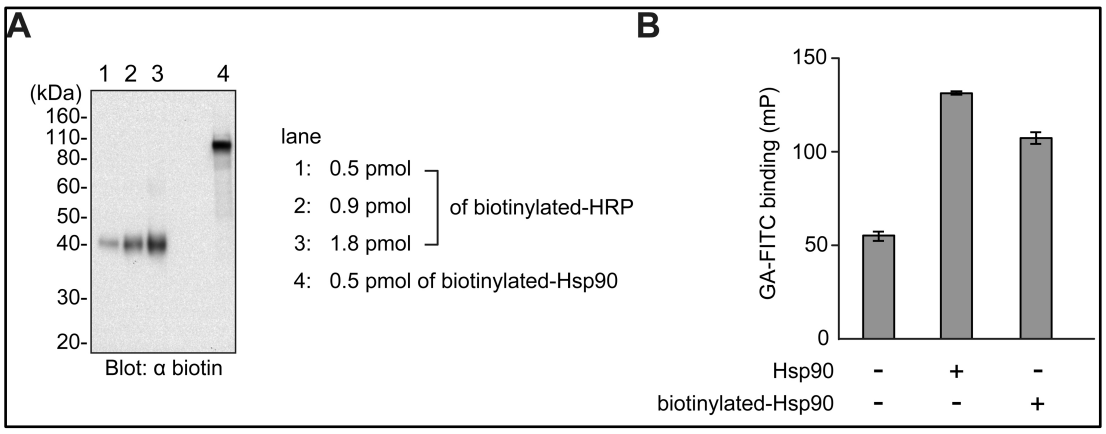


Figure S9. (**A**) Quantification of biotin conjugation to Hsp90. (**B**) Comparison of geldanamycin (GA)-binding avidity of Hsp90 and biotinylated-Hsp90 by FP assay.

Biopanning

Biopanning was performed as described previously with minor modifications. Biotinylated-Hsp90 (20 pmol) was immobilized on streptavidin-coupled magnetic beads (FG beads, Tamagawa Seiki, Japan). For biopanning, approximately 1.0×10^{11} PFU of T7Select10 library (-S-G-G-G-X_3-C-X_7-C-X_3; X represents any randomized amino acid) was modified via the 10BASE$_d$-T. After the modification, T7 phage library was suspended in selection buffer (PBS supplemented with 1% (v/v) TritonX-100 and 1% (w/v) BSA). To remove non-specific binders (i.e. beads and streptavidin binders), the modified T7 phage library was pre-incubated with streptavidin-coupled FG beads for 2 hours at 4 °C, and then the supernatant was further incubated with the Hsp90-immobilized beads for 12 hours. The latter beads were washed three times with each 200 μL of the selection buffer. Whole binding and washing process were performed using an automated machine (Target Angler 8, Tamagawa Seiki, Japan). Hsp90-bound phage was directly infected and amplified with *E. coli* BLT5403 strain. Stringent conditions were stepwisely applied to each round by shortening the binding time and by increasing the washing frequency. After the 6 rounds of the biopanning, randomly chosen T7 phage monoclones were subjected to DNA sequencing.

Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed as described previously with minor modifications. Each wells of streptavidin-conjugated 96-well plate (Nunc Immobilizer Streptavidin F96, Thermo Scientific) were coated with blocking buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% (v/v) Tween-20, and 0.5% (w/v) BSA) at 4 °C overnight. After washing with Tris-buffed saline, biotinylated-Hsp90 (3 pmol) was immobilized on each well. Approximately 2.0×10^{10} PFU of T7 phage in TBST was applied to the well and incubated for 1 hour at 25 °C with shaking by using a maximizer (MBR-022UP, TAITEC, Japan). The plate was washed three times with Tris-buffered saline supplemented with 0.5% (v/v) TritonX-100, and then Hsp90-bound phage was incubated with T7 tail fiber monoclonal antibody (1:5,000 dilution, Merck Millipore) and anti-mouse IgG HRP-linked antibody (1:5,000 dilution, Cell Signaling). After

washing with the TBST, o-phenylenediamine dihydrochloride substrate (SigmaFast OPD, Sigma Aldrich) was added, and the absorbance was quantified using a microplate reader equipped with a 450 nm band-pass filter (Bio-Rad).

Preparation of GST-fused Hsp90 domains and GST pull-down assay

Three domains of human Hsp90α, N-terminal (NTD: 9-236), middle (MD: 272-617), and C-terminal domains (CTD: 629-732) were prepared as described previously. Briefly, pGEX-4T-3 vector encoding fragments of *hsp90* gene (a gift from Dr. Franz-Ulrich Hartl, Max-Planck-Institute of Biochemistry, Germany) was introduced into *E. coli* BL21 (DE3) strain. Transformants were precultured overnight at 37 °C in 2 mL of LB medium supplemented with 100 μg/mL ampicillin, and then transferred to a 150 mL of fresh LB medium. After incubation for 4 hours at 37 °C, isopropyl β-D-1-thiogalactopyranoside was added at a final concentration of 0.2 mM, and the cells were further cultured for 20 hours at 20 °C. The cells were harvested, and suspended in ice-cold lysis buffer (50 mM Tris-HCl, pH 8, 300 mM NaCl, 1 mM EDTA, 5 mM 2-mercaptoethanol, 0.5 % (w/v) Triton X-100, and 1 × complete protease inhibitor cocktail minus EDTA). After cells disruption by ultrasonication, the crude cell extract was cleared by centrifugation at 20,000 × *g* for 10 min at 4 °C. Using a 0.42 μm membrane filter, the extract was further cleared. Supernatant was incubated with glutathione sepharose 4B (GE Healthcare) for 2 hours at 4 °C. After several washing with the lysis buffer, the sepharose was suspended in stock buffer (20 mM Tris-HCl, pH 8, and 50% (v/v) glycerol) and stored at -80 °C until use.

For GST pull-down assay, 20 μL (50% slurry) of the GST-Hsp90-immobilized sepharose was suspended in 200 μL of binding buffer (50 mM Tris-HCl, pH7.5, 150 mM NaCl, 0.05% (v/v) Tween-20, and 0.5% (w/v) BSA), and then 200 μL of the same buffer containing modified-T7 phage monoclone was added. After incubation for 2 hours at 4 °C, the sepharose was washed three times with Tris-buffered saline supplemented with 0.5% (v/v) Triton X-100. GST-fusion proteins were eluted with elution buffer (50 mM HEPES-KOH, pH 7.4, 100 mM NaCl, 1 mM DTT, and 50 mM

reduced glutathione). After addition of 4 × sample buffer (250 mM Tris-HCl, pH 6.8, 40% glycerol, 8% SDS, 20% 2-mercaptoethanol, and 0.008% bromophenol blue), the solution was incubated at 95 °C for 5 min.

SDS-PAGE and Western blot analysis

SDS-PAGE and Western blot analysis were performed as reported previously. Proteins were resolved by a 10% polyacrylamide gel. For Western blotting, proteins in the gel were transferred onto a polyvinylidene difuoride membrane (Bio-Rad). The blots were incubated with primary antibody, followed by incubation with anti-mouse IgG horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology). After several washes, the blots were incubated with ECL plus reagent (GE Healthcare Life Sciences), and detected using ChemiDoc XRS+ (Bio-Rad). Image contrast and brightness were adjusted in Photoshop CS4 (Adobe). Primary antibodies: anti-T7 tag mouse monoclonal antibody (Merck Millipore) and anti-FLAG mouse monoclonal antibody (M2, Sigma Aldrich). Note that antigen (M-A-S-M-T-G-G-Q-Q-M-G) of the anti-T7 tag mouse monoclonal antibody is N-terminal region of gp10, which is a component of bacteriophage T7.

Synthesis of Hsp90 NTD-binding crown molecule

A peptide (H_2N-R-S-W-C-R-K-S-R-K-N-S-G-G-G-L-V-W-C-F-OH) was synthesized and characterized by HiPep Laboratories (Japan). Purity of the peptide was estimated to be above 90%. For cyclization of EBB, the peptide was dissolved in phosphate buffer (10 mM phosphate-KOH, pH 7.4) at a final concentration of 100 μM, and then EBB (500 μM) and neutralized TCEP (500 μM) were added. The mixture was incubated overnight at 37 °C in the dark with shaking, and then lyophilized to reduce the solution volume. The lyophilizate was dissolved in 1% formic acid aqueous solution, and the crown molecule was purified with reverse-phase HPLC (Shimadzu, apan) equipped with a XTerra Prep MS C18 column (10 × 50 mm, Waters). The crown molecule was separated using a 0-100% linear gradient of acetonitrile containing 0.1%

trifluoroacetic acid during 12 min at a flow rate of 4 ml per a minute. The crown molecule was lyophilized and characterized by LC-MS (see below; Fig. S10).

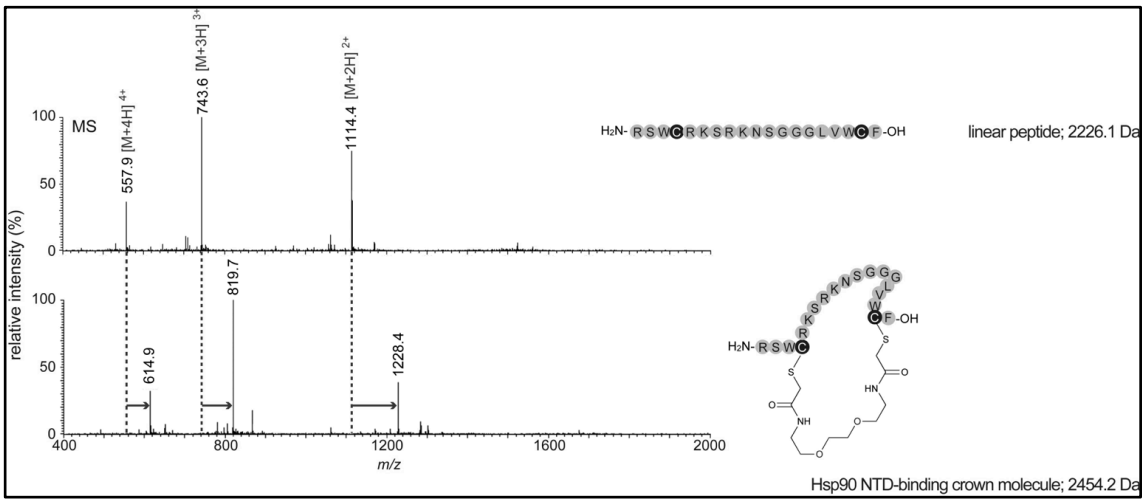


Figure S10. MS spectra of synthetic peptide and Hsp90 NTD-binding crown molecule.

Circular dichroism (CD) spectroscopy

All compounds were dissolved in phosphate buffer (20 mM phosphate-KOH, pH 7.2) at a final concentration of 50 μM. Circular dichroism spectra were recorded on a CD spectrometer (J-720W, JASCO, Japan) at 25 °C using a 0.2 cm path-length quartz cell (see below; Fig. S10). For reduction of disulfide-bond, TCEP was added at a final concentration of 1 mM.

Isothermal titration calorimetry (ITC)

ITC experiment was performed using MicroCal iTC_{200} (GE Healthcare). GST-tagged Hsp90 NTD was enriched using an ultrafiltration column (vivaspin column 500 MWCO 10 kDa, GE Healthcare), and buffer was changed to phosphate buffer (20 mM phosphate-KOH, pH 7.2, 50 mM NaCl, 1 mM TCEP). Protein concentration was determined by Bradford assay. For titration experiment, GST-Hsp90 NTD and compounds were diluted into 5 μM and 200 μM, respectively. Titrations were performed at 25°C. Injection parameters were the following: 2 μL volume, 4 sec

duration, 4 sec spacing, and 5 sec filter period. Reference power was set to 10 μcal/s. Data were analyzed with Origin software 7.0 (MicroCal). Curve fitting was performed using 1:1 interaction model.

Fluorescence polarization (FP) assay

Fluorescence polarization was measured using a HYBRID-3000ES (Photoscience, Japan) equipped with appropriate filters (Ex. 480 nm and Em. 535 nm). Instrument was operated in static mode. FITC-labeled geldanamycin (GA-FITC; 4 pmol, 20 nM) was incubated with various concentrations of GST-fused Hsp90 domains (NTD, MD, and CTD) in phosphate-buffered saline supplemented with 1 mM TCEP for 5 min at 30°C, and fluorescence polarization was measured. In the TCEP-containing solution, geldanamycin (GA) bound to Hsp90 in a short incubation time (*ca.,* 2 min; data not shown). Klotz plot was generated by GraphPad Prism software 6.0 (GraphPad Software), and the sigmoid curve was fitted with non-linear least squares analysis for K_D determination. For competition assay, various concentration of compounds were pre-incubated with GST-Hsp90 NTD (110 pmol, 550 nM) in phosphate-buffered saline supplemented with 1 mM TCEP for 10 min at room temperature. After addition of GA-FITC (4 pmol, 20 nM), the mixture was incubated for 5 min at 30 °C, and then fluorescence polarization was measured. GA-FITC and geldanamycin were purchased from Enzo Life Sciences (cat No. BML-EI361-0001) and StressMarq (cat No. SIH-111A/B), respectively. Concentrations of GA-FITC and geldanamycin were determined by absorption coefficient at 336 nm.

■ 4.4 References

[1] J. Nishimura, Y. Nakamura, Y. Hayashida and T. Kudo, *Acc. Chem. Res.*, 2000, **33**, 679-686.

[2] H. Murakami, A. Kawabuchi, R. Matsumoto, T. Ido and N. Nakashima, *J. Am. Chem. Soc.*, 2005, **127**, 15891-15899.

[3] M. X. Wang, *Acc. Chem. Res.*, 2012, **45**, 182-195.

[4] S. B. Nimse and T. Kim, *Chem. Soc. Rev.*, 2013, **42**, 366-386.

[5] S. J. Dalgarno, K. M. Claudio-Bosque, J. E. Warren, T. E. Glass and J. L. Atwood, *Chem. Commun.*, 2008, 1410-1412.

[6] B. Koszarna and D. T. Gryko, *Chem. Commun.*, 2007, 2994-2996.

[7] R. S. Czernuszewicz, V. Mody, A. Czader, M. Galezowski and D. T. Gryko, *J. Am. Chem. Soc.*, 2009, **131**, 14214-14215.

[8] M. Taki, S. Sugita, Y. Nakamura, E. Kasashima, E. Yashima, Y. Okamoto and J. Nishimura, *J. Am. Chem. Soc.*, 1997, **119**, 926-932.

[9] C. J. Pedersen, *J. Am. Chem. Soc.*, 1967, **89**, 7017-7036.

[10] R. Nonokawa and E. Yashima, *J. Am. Chem. Soc.*, 2003, **125**, 1278-1283.

[11] M. Bayrakcı, E. Maltaş, S. Yiğiter and M. Özmen, *Macromol. Res.*, 2013, **21**, 1029-1035.

[12] P. Monvisade, P. Hodge and C. L. Ruddick, *Chem. Commun.*, 1999, 1987-1988.

[13] K. Fukunaga, T. Hatanaka, Y. Ito and M. Taki, *Mol. BioSyst.*, 2013, **9**, 2988-2991.

[14] S. Chen, J. Touati and C. Heinis, *Chem. Commun.*, 2014, DOI: 10.1039/c3cc47496h.

[15] T. Hamamoto, M. Sisido, T. Ohtsuki and M. Taki, *Chem. Commun.*, 2011, **47**, 9116-9118.

[16] M. Minami, M. Nakamura, Y. Emori and Y. Minami, *Eur. J. Biochem. / FEBS*, 2001, **268**, 2520-2524.

[17] Y. Suzuki, M. Minami, M. Suzuki, K. Abe, S. Zenno, M. Tsujimoto, K. Matsumoto and Y. Minami, *J. Biol. Chem.*, 2009, **284**, 35597-35604.

[18] F. Shinozaki, M. Minami, T. Chiba, M. Suzuki, K. Yoshimatsu, Y. Ichikawa, K. Terasawa, Y. Emori, K. Matsumoto, T. Kurosaki, A. Nakai, K. Tanaka and Y. Minami, *J. Biol. Chem.*, 2006, **281**, 16361-16369.

[19] F. U. Hartl, A. Bracher and M. Hayer-Hartl, *Nature*, 2011, **475**, 324-332.

[20] M. Taipale, D. F. Jarosz and S. Lindquist, *Nat. Rev. Mol. Cell Biol.*, 2010, **11**, 515-528.

[21] M. A. Biamonte, R. Van de Water, J. W. Arndt, R. H. Scannevin, D. Perret and W. C. Lee, *J. Med. Chem.*, 2010, 53, 3-17.

[22] J. Trepel, M. Mollapour, G. Giaccone and L. Neckers, *Nat. Rev. Cancer*, 2010, **10**, 537-549.

[23] T. Hatanaka, S. Ohzono, M. Park, K. Sakamoto, S. Tsukamoto, R. Sugita, H. Ishitobi, T. Mori, O. Ito, K. Sorajo, K. Sugimura, S. Ham and Y. Ito, *J. Biol. Chem.*, 2012, **287**, 43126-43136.

[24] C. E. Stebbins, A. A. Russo, C. Schneider, N. Rosen, F. U. Hartl and N. P. Pavletich, *Cell*, 1997, **89**, 239-250.

[25] J. C. Young, C. Schneider and F. U. Hartl, *FEBS Lett.*, 1997, **418**, 139-143.

[26] T. Zhang, A. Hamza, X. Cao, B. Wang, S. Yu, C. G. Zhan and D. Sun, *Mol. Cancer Ther.*, 2008, **7**, 162-170.

■ Concluding remarks

Post-translational chemical modification of a genetically-encoded peptide library is an useful method for construction of hybrid molecule libraries possessing arbitrary functions (*e.g.,* fluorescence). My study stands on this method. Current studies are employing the M13 phage display peptide library for the post-translational chemical modification, however the M13 library has intimate problems (*e.g.,* sequence bias, infectivity loss associated with the modification etc.). To address the problem, a novel method for the construction of the hybrid molecule library is established in this study. By using the simple thioetherification reaction on bacteriophage T7-displayed peptides, three different characters of the hybrid molecule libraries have been constructed. Additionally, target-specific binders have been successfully obtained from these libraries. Among these, Hsp90-binding macrocycle has unique and interesting properties: the macrocycle specially binds to the N-terminal domain (NTD) of Hsp90, and can enhance binding of geldanamycin to the Hsp90 NTD. Though the binding affinity of the macrocycle is far enough (K_D = 1.7 μM), a potential novel strategy of Hsp90 inhibition is presented.

In the future, I will construct a novel peptide-based hybrid library and screen Hsp90-specific binders possessing druggable properties (i.e., higher target-binding affinity, higher specificity against a target, stronger proteolysis resistance, good cell penetrability, and good water solubility etc.).

■ List of publications

Chapter 1

Fukunaga, K. and Taki, M., Practical Tips for Construction of Custom Peptide Libraries and Affinity Selection by Using Commercially Available Phage Display Cloning Systems. ***J. Nucl. Acids,*** 2012, 2012, 295719.

Chapters 2-3

Fukunaga, K., Hatanaka, T., Ito, Y. and Taki, M. Gp10 based-thioetherification (10BASE$_d$-T) on a displaying library peptide of bacteriophage T7. ***Mol. BioSyst.,*** 2013, 9, 2988-2991.

Fukunaga, K., Hatanaka, T., Ito, Y. and Taki, M. An Artificial Molecule-Peptide Hybrid Discovered via the 10BASE$_d$-T Binds to Substrate-Binding Site of Glutathione *S*-Transferase. ***Pept. Sci. 2013,*** 2014, in press.

Chapter 4

Tokunaga, Y., Azetsu, Y., **Fukunaga, K.**, Hatanaka, T., Ito, Y. and Taki, M. Pharmacophore Generation from a Drug-like Core Molecule Surrounded by a Library Peptide via the 10BASE$_d$-T on Bacteriophage T7. ***Molecules***, 2014, 19, 2481-2496.

Tokunaga, Y., **Fukunaga, K.**, Hatanaka, T., Ito, Y. and Taki, M. Selection of Streptavidin-Binding Artificial Peptide Possessing Salicylic Acid Moiety via the 10BASE$_d$-T on the Bacteriophage T7. ***Pept. Sci. 2013,*** 2014, in press.

Chapter 5

Fukunaga, K., Hatanaka, T., Ito, Y., Minami, M. and Taki, M. Construction of a crown ether-like supramolecule library by conjugation of genetically-encoded peptide linkers displayed on bacteriophage T7. ***Chem. Commun. (Camb.)***, 2014, 50, 3921-3923.

* **This article was selected as an "inside front cover".**

Others

Fukunaga, K., Kudo, T., Toh-e, A., Tanaka, K. and Saeki, Y. Dissection of the assembly pathway of the proteasome lid in Saccharomyces cerevisiae. ***Biochem. Biophys. Res. Commun.***, 2010, 396, 1048-1053.

Kim, S., Saeki, Y., **Fukunaga, K.**, Suzuki, A., Takagi, K., Yamane, T., Tanaka, K., Mizushima, T. and Kato, K. Crystal Structure of Yeast Rpn14, a Chaperone of the 19S Regulatory Particle of the Proteasome. ***J. Biol. Chem.***, 2010, 285, 15159-15166.

Saeki, Y., **Fukunaga, K.** and Tanaka, K. Proteasome inhibitors. ***Nihon Rinsho***, 2010, 68, 1818-1822.
[Article in Japanese]

Sakata, E., Stengel, F., **Fukunaga, K.**, Zhou, M., Saeki, Y., Förster, F., Baumeister, W., Tanaka, K. and Robinson, C. V. The catalytic activity of Ubp6 enhances maturation of the proteasomal regulatory particle. ***Mol. Cell***, 2011, 42, 637-649.

Saeki, Y., and **Fukunaga, K.** Overview of the 26S proteasome assembly. ***Jikken Igaku***, 2011, 29, 1868-1874.
[Article in Japanese]

Fukunaga, K. and Taki, M. Construction of a Peptide Expression Vector Library for Phenotypic Screening of Bioactive Peptides in Yeast. ***Pept. Sci. 2013,*** 2014, in press.

■ List of presentations

1. **Fukunaga, K.**, Saeki, Y. and Tanaka, K.
   "Exploration of chaperone, which assists assembly of the 26S proteasome lid in *Saccharomyces cerevisiae*."
   The 32th annual meeting of the molecular society of Japan, Yokohama, Dec. 2009, Poster presentation.

2. **Fukunaga, K.**, Saeki, Y. and Tanaka, K.
   "A quantitative proteomic analysis of tUb-binding proteins with different length in yeast."
   1st conference on proteomics of protein degradation & ubiquitin pathways, Vancouver, Jun. 2010, Poster presentation.

3. **Fukunaga, K.**, Kudo, T., Toh-e, A., Tanaka, K. and Saeki, Y.
   "Assembly of the 26S proteasome lid in *Saccharomyces cerevisiae*.
   The 43th annual meeting of the yeast genetics society of Japan, Nara, Sep. 2010., Poster presentation.

4. **Fukunaga, K.**, Kudo, T., Toh-e, A., Tanaka, K. and Saeki, Y.
   "Assembly pathway of the proteasome lid in yeast."
   Biochemistry and molecular biology 2010, Kobe, Dec. 2010, Poster presentation.

5. Kudo, T., **Fukunaga, K.**, Tanaka, K. and Saeki, Y.
   "A proteomic analysis of ubiquitin-binding proteins by uncleavable polyubiquitins in yeast."
   Biochemistry and molecular biology 2010, Kobe, Dec. 2010, Poster presentation.

6. Saeki, Y., **Fukunaga, K.**, Mizushima, T., Sakata, E., Baumeister, W. and Tanaka, K.
   "Assembly, structure, and function of the 26S proteasome."
   1st Korea-Japan symposium on protein metabolism, Seoul, Jan. 2011, Oral presentation.

7. **Fukunaga, K.**, Sakata, E., Saeki, Y. and Tanaka, K.
"Ubp6 positively regulates assembly of the proteasome.
The 44th annual meeting of the yeast genetics society of Japan, Fukuoka, Sep. 2011, Oral presentation.

8. Saeki, Y., **Fukunaga, K.**, Sakata, E., Baumeister W. and Tanaka, K.
"Rad23 and Ubp6 are involved in 26S proteasome assembly."
The 84th annual meeting of the Japanese biochemical society, Kyoto, Sep. 2011, Oral presentation.

9. **Fukunaga, K.**, Sakata, E., Stengel, F., Saeki, Y., Baumeister, W., Robinson, C. V. and Tanaka, K.
"Ubp6 and Rad23 are involved in the proteasome assembly."
3rd EMBO conference on ubiquitin and ubiquitin-like modifiers, Dubrovnik, Sep. 2011, Poster presentation.

10. Yukii, H., Saeki, Y., Pack, C., **Fukunaga, K.**, Sakata, E., Sako, Y., Baumeister, W. and Tanaka, K.
"The 26S proteasome completes its assembly in the cytosol prior to enter the nucleus in yeast."
3rd EMBO conference on ubiquitin and ubiquitin-like modifiers, Dubrovnik, Sep. 2011, Poster presentation.

11. Saeki, Y., Kim, S., Toh-e, A., Sakata, E., **Fukunaga, K.**, Yukii, H., Sako, Y. and Tanaka, K.
"The 26S proteasome completes its assembly process in the cytoplasm prior to the nuclear translocation."
The 34th annual meeting of the molecular biology society of Japan, Yokohama, Dec. 2011, Poster presentation.

12. Abiko, D., **Fukunaga, K.** and Taki, M.
"Functionalization of bacteriophage T7-displayed peptides by chemical modification: construction of fluorescent peptide library and exploration of GST

inhibitor”
The 25th summer school of young associations in biofunctional chemistry, Tokyo, 26 Jul. 2013, Poster presentation.

13. Tokunaga, Y., **Fukunaga, K.** and Taki, M.
“Chemical evolution of bacteriophage T7-displayed peptides: construction of PEG cross-linked library and exploration of Hsp90 inhibitor”
The 25th summer school of young associations in biofunctional chemistry, Tokyo, 26 Jul. 2013, Poster presentation.

14. **Fukunaga, K.** and Taki, M.
“Gp10 based-thioetherification (10BASE$_d$-T) on a displaying peptide of the bacteriophage T7.”
10th australian international peptide conference, Penang, 11 Sep. 2013, Poster presentation.
* **This travel was supported by travel awards from Japan peptide society and yoshida foundation for science and technology - to K.F.**

15. **Fukunaga, K.** and Taki, M.
“Construction of artificial peptide library on bacteriophage T7.”
7th bio-related chemistry symposium, Nagoya, 29 Oct. 2013, Oral presentation.

16. **Fukunaga, K.**, Hatanaka, T., Ito, Y., Minami, M. and Taki, M.
“Chemical evolution of bacteriophage T7-displayed peptides.”
4th Asia-Pacific international peptide symposium, Osaka, 6 Nov. 2013, Oral presentation.
* **This oral presentation was awarded the good stone award for young scientists.**

17. Tokunaga, Y., **Fukunaga, K.**, Hatanaka, T., Ito, Y. and Taki, M.
“Pharmacophore generation from a drug-like molecule surrounded by library peptide via the 10BASE$_d$-T.”
4th Asia-Pacific international peptide symposium, Osaka, 6 Nov. 2013, Poster

presentation.

18. **Fukunaga, K.** and Taki, M.
"Exploration of bioactive peptides from random peptide library expressed in yeast cells"
4th Asia-Pacific international peptide symposium, Osaka, 7 Nov. 2013, Poster presentation.

■ List of grants and awards

Grants

- Sasakawa scientific research grant from the Japan science society, FY2013
  "Explorative study of novel bioactive peptides in yeast."

- Travel award from yoshida foundation for science and technology, FY2013

- Sasakawa scientific research grant from the Japan science society, FY2014
  "Identification of *Mycobacterium tuberculosis* Pup ligase-recognizing motif and application to construction of a cyclic peptide library."

Awards

- Exemption from return of a student grant by outstanding achievements
  Japan Student Services Organization, Jun. 2011

- Travel award from the Japan peptide society, FY2013
  "Gp10 based-thioetherification (10BASE$_d$-T) on a displaying peptide of bacteriophage T7."

- Good stone award for young scientist's oral presentation
  4th Asia-Pacific international peptide symposium, Osaka, 7 Nov. 2013
  "Chemical evolution of bacteriophage T7-displayed peptides."

■ Acknowledgements

I would like to express my sincerest gratitude to professor Dr. Masumi Taki (UEC) for giving me an opportunity to work in his laboratory. His constant support, faith and encouragement have been extremely helpful for development of this work and for my life as a scientist. I would also like to thank all members of his laboratory.

I am most grateful to professor Dr. Michiko Minami (Tokyo Gakugei University) for her constant encouragement.

I am grateful to professor Dr. Yuji Ito and Dr. Takaaki Hatanaka (Kagoshima University) for providing T7 phage peptide libraries, professor Dr. Franz-Ulrich Hartl (Max-Planck-Institute of Biochemistry) for providing plasmids, the late professor Dr. Yasufumi Minami (Maebashi Institute of Technology) for providing a protein, professor Dr. Kazunori Ikebukuro and professor Dr. Wataru Yoshida (Tokyo University of Agriculture and Technology) for use of an ITC instrument, Mr. Tomoki Kiuchi (Okayama University), Mr. Yuuki Tokunaga, Mr. Yuuki Azetsu, and Ms. Seiko Yamada (UEC) for technical assistance, and Dr. Hiroshi Kita (Kaneka Co., Ltd.) and Dr. Aoi Shiraishi (Institute for Antibodies Co., Ltd.) for valuable suggestions.

Financially, this work was supported by a research and development projects of the Industrial Technology Research Grant Program in the New Energy and Industrial Technology Development Organization (NEDO), by grants of Japan Society for the promotion of Science - Grant-in-Aid for Young Scientists (A) and for Challenging Exploratory Research - to Dr. Masumi Taki.