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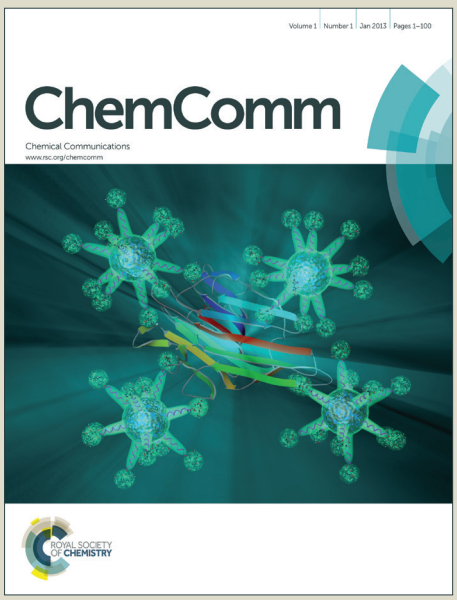

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Construction of a crown ether-like supramolecular library by conjugation of genetically-encoded peptide linkers displayed on bacteriophage T7

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By using the 10BASE$_d$-T, we have synthesized a crown ether-like macrocyclic library possessing randomized peptide linker on bacteriophage T7. Among 1.5 × 10^9 diversity of the supramolecule candidates, we have obtained a specific binder for the N-terminal domain of Hsp90.

Man-made macrocyclic molecules of diverse types (*e.g.,* cyclophanes,[1] rotaxanes,[2] calixarenes,[3-5] porphyrinoids,[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 its analogues[9,10] possess biocompatibility because hydrophilic oligoethylene structure increases water solubility, hence prevents aggregation. The ether oxygen atoms would form hydrogen bonds 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 such 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 not enough.

Recently, we 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 we cyclized both ends of oligoethyleneglycol unit by randomized peptide linker via the thioether linkage, to afford the crown analogue library with vast diversity (i.e., 10^9).

We synthesized a cysteine-reactive bifunctional synthon, *N,N'*-[1,2-ethanediyl-oxy-2,1-ethanediyl]bis(2-bromoacetamide) (EBB; Fig. 1A) as the core of diazacoronands, and the 10BASE$_d$-T was performed as follows (Fig. 1B) : T7 phage-displayed peptides (1.0 × 10^{11} plaque forming units) 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, we 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

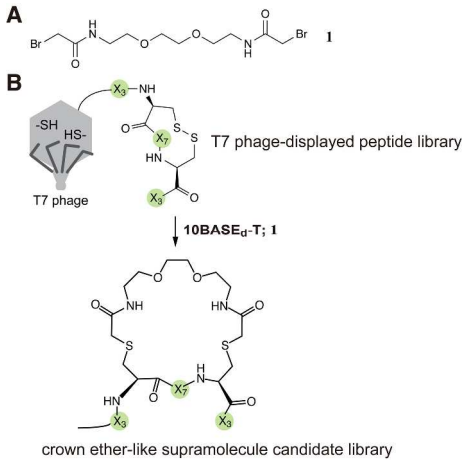

Fig. 1 (A) Chemical structure of EBB (**1**). (B) Construction of a crown ether-like supramolecule candidate library through the 10BASE$_d$-T. X represents randomized amino acid.

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, †ESI). Generally, acquisition of a MS/MS spectrum of a ring-forming peptide region is difficult.[14,15] However, we tried in-depth mass spectrometric analysis of the crown analogue on T7 phage, to clarify the amino acid sequence of the ring-forming peptide region. Here we performed a LC-MS/MS analysis of the macrocycle by enzymatic digestion of the ring-forming peptide (Fig. 2A). 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 the amino acid sequence inside the ring (Fig. 2B). Consequently, the model crown analogue on T7 phage was unambiguously identified.

As a target of biopanning using the crown analogue library, we used heat shock protein 90 (Hsp90), which plays a crucial role in protein homeostasis, cell signaling, and stress response supported by our previous results.[16,17] Hsp90 is a highly conserved molecular chaperone, which governs cellular protein quality control,[18,19] and considered as an important class of drug target in cancer therapy.[20,21] In advance of 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)[13,22] via the 10BASE$_d$-T as shown in Fig. 1B. We next examined the infectivity of the modified T7 phage library by plaque assay, and confirmed that the infectivity titer is fully retained (Fig. S2, †ESI).

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, †ESI). 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, †ESI). 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, we speculate that trace amounts of the crown analogue with the longer peptide linker, which was encoded by misligated DNA fragments (Fig. S4, †ESI), were enriched by biopanning. To confirm the effect of a crown moiety for the binding, biopanning using the *naïve* cyclic peptide library with a conventional disulfide (S-S) bridge (Fig. 1B, upper) was also performed. By sequence analyses, the 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. S5B, †ESI). 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 candidate library; only N-terminal arginine and C-terminal hydrophobic aromatic amino acids (tryptophan) were identical. This

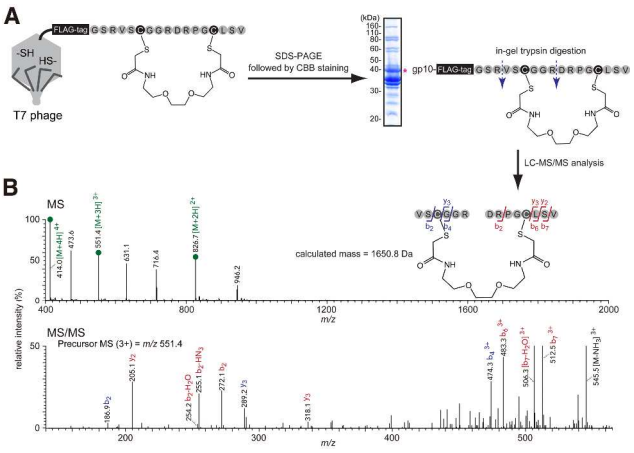

Fig. 2 (A) Strategy for identification of a model crown analogue displayed on T7 phage. A T7 phage-displaying model linker peptide was conjugated with EBB via the 10BASE$_d$-T, and then separated into a subunit by SDS-PAGE. After coomassie brilliant blue (CBB) staining, the magenta asterisk corresponding to the crown-fused gp10 was excised and subjected to in-gel trypsinization followed by LC-MS/MS analysis. Blue arrows indicate the trypsin cleavage site. (B) MS analysis of the ring-opening crown analogue. Above panel: MS spectrum. A series of multiple charged ions (green 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: DQAAYLAPGENLDDKRK, *m/z* 716.4: DLALER). Lower panel: MS/MS spectrum. Trypsin could not cleave arginine C-terminus before proline as reported previously.[13]

indicates that the crown moiety strongly affects the geometry of the whole macrocycle structure of the *naïve* library, to generate a novel supramolecule candidate library.

We next 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[19] (Fig. 3A). Using each glutathione *S*-transferase (GST)-fused Hsp90 domain,[23] GST-pull down assay was carried out. We found that the crown ether analogue on the T7 phage monoclone exclusively bound to the NTD of Hsp90 (Fig. 3A). To determine affinity of the Hsp90 NTD-binding crown analogue, we synthesized the linker peptide with solid-phase peptide synthesis followed by cyclization with an 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. 3B). This suggests that both structures of the crown moiety and the rest of the 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. S6, †ESI). This structural flexibility of the crown analogue might be important in the interaction, because Hsp90 recognizes unfolded substrates.[18]

Geldanamycin (GA) is an anticancer natural product, which binds to the ATP-binding pocket on the NTD of Hsp90.[20,21] We

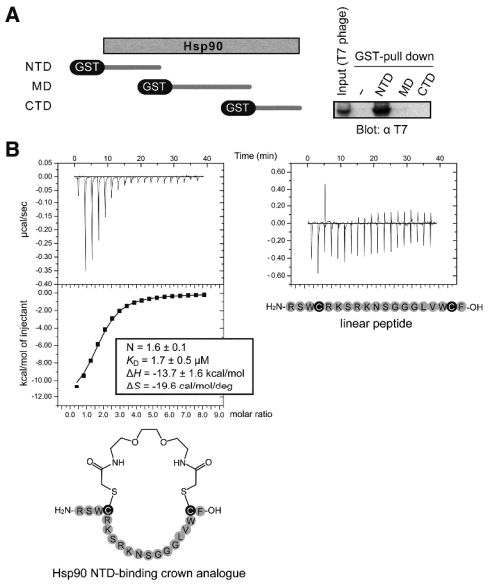

Fig. 3 (A) GST pull-down assay. EBB-modified T7 phage monoclone (-R-S-W-C*-R-K-S-R-K-N-S-G-G-G-L-V-W-C*-F; *Cys were conjugated with an EBB core) was used as the input. N-terminal (NTD), middle (MD), and C-terminal (CTD) domains of Hsp90 are schematically shown on the left. (B) Isothermal titration calorimetry 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, Δ*H*: enthalpy change, Δ*S*: entropy change.

here examined whether the crown analogue competes with GA for the Hsp90 NTD-binding. Using a fluorescein-5-isothiocyanate labelled GA (GA-FITC), a 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. S7B, †ESI). 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.[20] 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.[24]

In conclusion, we demonstrated the first example of a new approach towards construction of a macrocyclic library as supramolecule candidates with vast diversity on T7 phage. From the library, we successfully found the Hsp90-binding supramolecule. Enthalpy change was strongly contributed to the interaction between the discovered crown analogue and Hsp90 NTD. We 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, we are now trying to construct artificial libraries of multicyclic structures to find supramolecules with greater binding affinity.

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Notes and references

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Table of contents entry