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Cu-free cycloaddition for identifying catalytic active adenylation domains of nonribosomal peptide synthetases by phage display

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ABSTRACT

To engineer the substrate specificities of nonribosomal peptide synthetases (NRPS), we developed a method to display NRPS modules on M13 phages and select catalytically active adenylation (A) domains that would load azide functionalized substrate analogs to the neighboring peptidyl carrier protein (PCP) domains. Biotin conjugated difluorinated cyclooctyne was used for copper free cycloaddition with an azide substituted substrate attached to PCP. Biotin-labeled phages were selected by binding to streptavidin.

Polyketides and nonribosomal peptides are two large classes of pharmaceutically important natural products constituting a rich source of anticancer, antifungal, antibiotic, and immunosuppressant reagents.[1] The biosynthesis of these natural products shares the same logic by stepwise chain elongation on multimodular polyketide synthases (PKS) or nonribosomal peptide synthetases (NRPS) as the enzymatic assembly lines of simple building blocks—carboxylic acids for PKS and amino acids for NRPS.[2–5] The acyltransferase (AT) domain in PKS is responsible for selecting a specific acyl-CoA as the substrate and attaching the acyl group to the free thiol at the end of the phosphopantetheinyl prosthetic group (Ppant) of the acyl carrier protein (ACP) domain.[6] Similarly the adenylation domain in NRPS is responsible for selecting a specific amino acid to be attached to the Ppant arm on the peptidyl carrier protein (PCP) domain embedded within the NRPS enzymes (Fig. 1A).[7,8] Subsequently, substrates loaded on the carrier proteins by AT and A domains are incorporated into the elongating polyketide or nonribosomal peptide chains by stepwise condensation reactions.

To overcome the difficulties in organic synthesis, several strategies have been developed for the biosynthesis of structural analogs of polyketides and nonribosomal peptides. One method, known as 'combinatorial biosynthesis', combines genes originated from different PKS and NRPS biosynthetic pathways in one bacterial host for the assembly of natural product molecules of new structures.[9–13] In another strategy called 'precursor directed biosynthesis', nonnative building block molecules are supplied to the culture media for their incorporation in the biosynthetic reactions catalyzed by PKS and NRPS enzymes.[14] Often the substrate tolerance of the biosynthetic enzymes limits the structural diversity of the natural product analogs that can be produced by biosynthesis. For examples, the AT domain in a PKS module and the A domain in a NRPS module act as the gate-keeping domains exhibiting strict specificity for their cognate acyl-CoA or amino acid substrates, respectively.[6–8] To achieve greater diversity for substrate loading in NRPS enzymes, we here report a phage selection method for the identification of the A domains with altered substrate specificity. This method can be potentially used for directed evolution of the AT and A gate-keeping domains in the PKS and NRPS enzymes to reprogram their substrate specificities.

Phage display establishes a direct linkage between the polypeptide entities displayed on the phage surface and the encoding gene encapsulated inside the phage particle.[15] In this way, phage display provides a general platform for quickly sorting through large libraries of peptides or proteins for the selection of desired binding[16,17] or catalytic activities.[18–22] To engineer the substrate specificity of the NRPS A domains by phage selection, we displayed the A-PCP didomain on the surface of M13 phages and used alkyne or azide functionalized substrate analogs for A domain catalyzed substrate loading on the neighboring PCP domain (Fig. 1B). Substrate analogs covalently attached to PCP can then be conjugated to biotin by Huisgen's 1,3-cycloaddion reaction between the azide and alkyne functionalities.[23,24] Such strategy directly couples substrate loading catalyzed by the A domain on the phage surface with biotin conjugation to the corresponding phage particles. Biotin-labeled phages can then be selected by binding to streptavidin.

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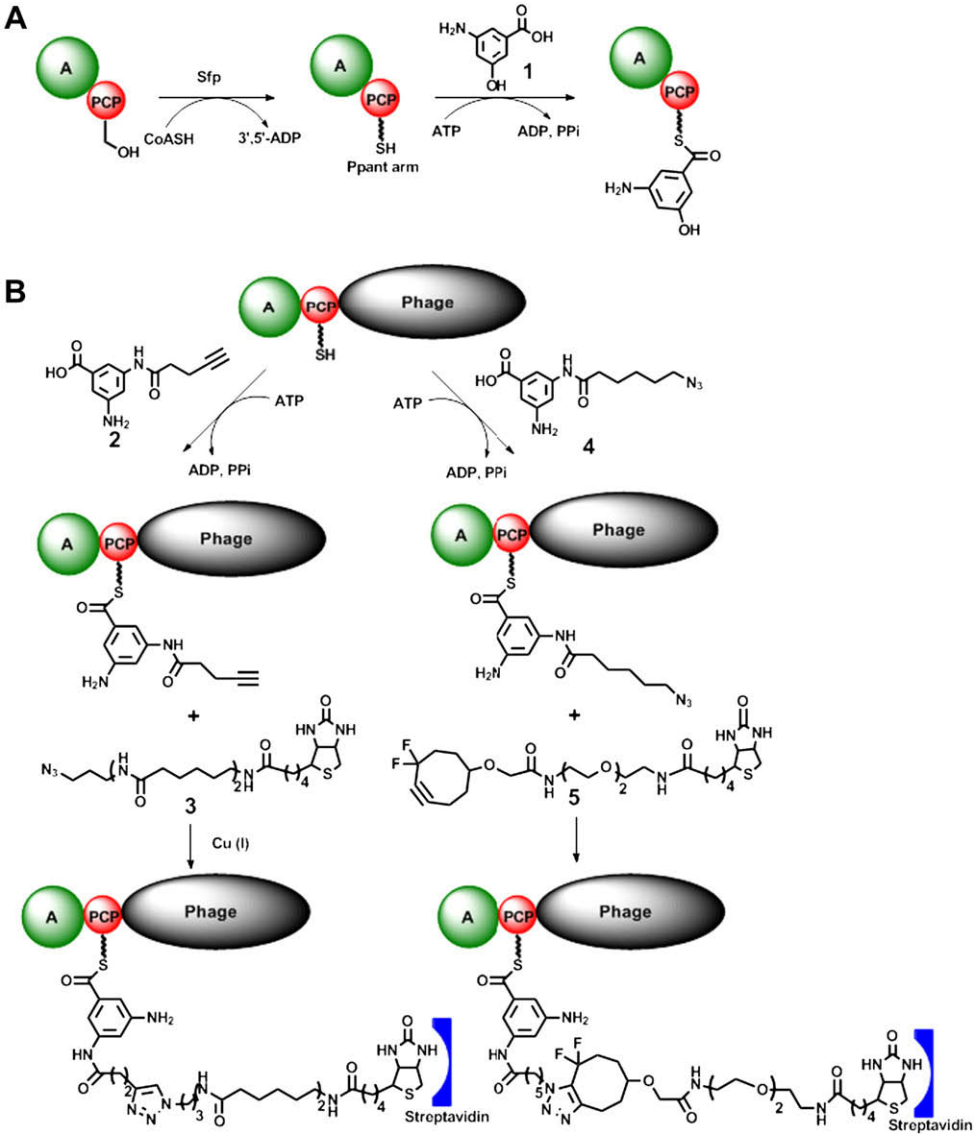

Figure 1. (A) Loading of AHB **1** on the A-PCP didomain of RifA LM after the PCP domain being activated by Sfp catalyzed Ppant modification. (B) Phage selection strategy for engineering the substrate specificity of the A domain of RifA LM.

To test this selection method, we cloned the gene encoding A-PCP didomain of RifA LM into phagemid vector pCom3H for the display of A-PCP on the surface of M13 phages as the N-terminal fusion to truncated phage capsid protein pIII.[25] RifA is the N-terminal component of rifamycin synthetase from *Amycolatopsis mediterranei* that is responsible for the biosynthesis of proansamycin X, a precursor to antibiotics rifamycin B.[26] RifA LM was previously cloned by Khosla and colleagues for expression and enzymatic studies.[27] To assay the display efficiency of the A-PCP didomain, we used Sfp phosphopantetheinyl transferase to label the PCP domain on the phage surface with biotin-CoA **6** for the attachment of biotin-Ppant group to a specific Ser residue on PCP.[28,29] Western blot of the labeling reaction mixture was probed with streptavidin–horseradish peroxidase (HRP) conjugate and showed a band of 84 kDa in size corresponding to A-PCP fused to pIII (Supplemental Fig. 1). This suggests that the A-PCP didomain from RifA LM can be displayed on the surface of M13 phages with good efficiency.

The A domain of RifA LM is specific for 3-amino-5-hydroxybenzoate (AHB) **1** and has been shown to have substrate tolerance for substituted benzoates at the 3 and 5 positions (Fig. 1A).[27,30] We thus synthesized AHB analogs **2** and **4** with alkyne or azide functionalities at the 3 position for their conjugation with biotin linked cycloaddition partners **3** and **5** after substrate loading on the A-PCP didomain displayed on phage surface (Fig. 1B). The reaction between **2** and **3** needs to be catalyzed by Cu(I) salt[34] while the reaction between **4** and **5** does not need the copper catalyst following the method developed by Bertozzi and colleagues using a strained alkyne functionality in cyclooctyne for azide conjugation.[31,32]

To assay the activities of **2** and **4** for substrate loading, the A-PCP didomain of RifA LM was expressed as C-terminal fusions to 6× His tag. ATP-pyrophosphate (PPi) exchange assay showed that both **2** and **4** can be taken as the substrates of the A domain with the alkyne analog **2** retaining 77% of the activity of the native substrate AHB **1** and the azide analog **4** 5% of the AHB activity (Supplemental Fig. 2). Covalent loading of **2** and **4** on PCP was also confirmed by Western blot (Supplemental Fig. 3). In this assay, A-PCP didomain was first activated by Sfp catalyzed Ppant modification of PCP for the formation of holo-A-PCP. After incubation with **2** and **4**, substrate loading on holo-A-PCP was detected by reacting biotin functionalized **3** and **5** with substrate analogs **2** and **4** attached to PCP, respectively. Western blot of the reaction mixture was probed with streptavidin–HRP and biotin conjugation

with the A-PCP didomain was confirmed by detecting a band of 67 kDa, matching the size of A-PCP of RifA LM (Supplemental Fig. 3). These results suggest that although substrate analogs **2** and **4** have lower activity then the native substrate **1**, both substrates can be loaded on PCP by A domain catalysis. We thus decided to test if **2** and **4** could be loaded on the A-PCP didomain displayed on phage surface for the selection of catalytic active A domains.

Compound **2** was incubated with phages displaying A-PCP didomain after Sfp catalyzed Ppant modification of PCP. After substrate loading, phage particles were precipitated by polyethylene glycol (PEG) followed by conjugation with biotin-azide **3** in the presence of CuBr (Fig. 1B). Western blot of the phage loading reaction showed a band corresponding to the size of A-PCP didomain fusion with the phage capsid protein pIII (Fig. 2A, lane 3). This suggests that biotin-azide **3** can be specifically conjugated with alkyne functionalized substrate **2** loaded on A-PCP didomain on the phage surface. Biotin conjugation to phage displayed A-PCP was dependent on substrate loading with **2** since no biotin conjugation was found when **2** was not included in the loading reaction or phage displayed A-PCP was not activated for Ppant modification (Fig. 2A, lanes 1 and 2). We then assayed if biotin-labeled phages from copper catalyzed cycloaddition reactions can be bound to streptavidin surface for affinity enrichment. Unfortunately we found that the efficiency of phage infectivity was decreased by more than 100-fold upon the phages being exposed to 10 μM CuBr or $CuSO_4$ for 30 min at room temperature. Under these conditions, phages would not be able to infect the *Escherichia coli* cells for phage amplification and subsequent rounds of selection after the cycloaddition reaction catalyzed by the copper salt.

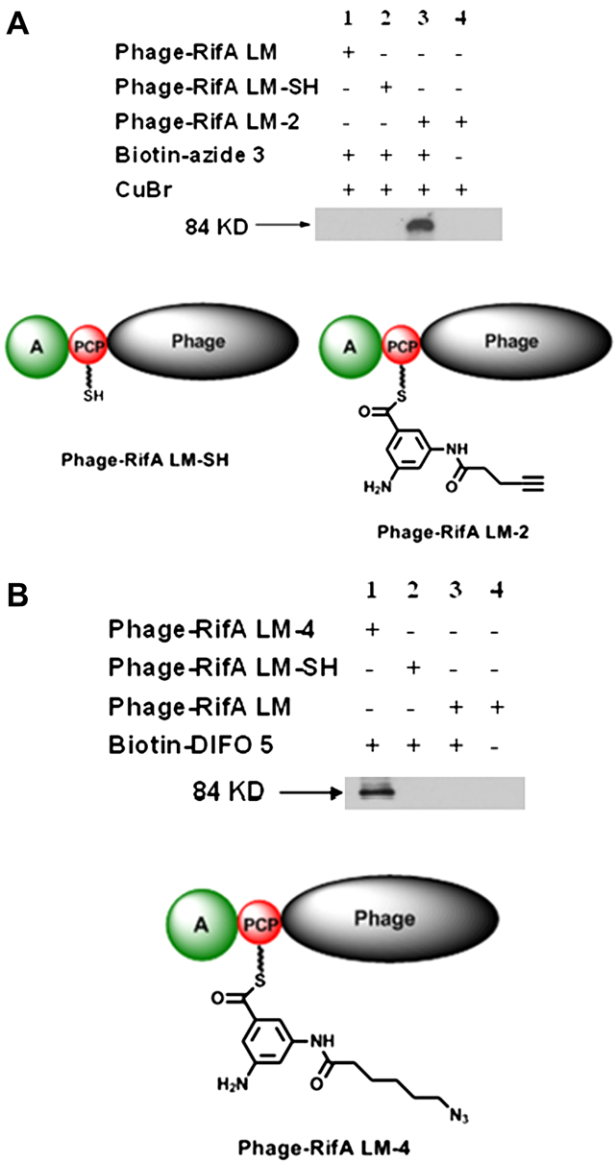

Figure 2. (A) Western blot of phage displayed A-PCP didomain of RifA LM after substrate loading with **2** and conjugation with biotin-azide **3**. (B) Western blot of phage displayed A-PCP didomain of RifA LM after substrate loading with **4** and conjugation with biotin-DIFO **5**. The Western blots were probed with streptavidin–HRP.

We then switched to azide substrate **4** for A-PCP loading on phage surface since the cycloaddition reaction between **4** and biotin-DIFO **5** does not require copper catalysts. Following the previous procedure, phage displayed holo-A-PCP of RifA was first loaded with **4** in the presence of ATP. After PEG precipitation to remove the free substrate analog in the reaction, **5** was added to the resuspended phage particles in copper free buffer and the coupling reaction was allowed to proceed for 4 h. Western blot of the reaction mixture showed biotin attachment to the A-PCP didomain fused to phage capsid protein pIII (Fig. 2B, lane 1). In contrast, control reactions without Ppant activation or without substrate loading with **4** did not show biotin attachment (Fig. 2B, lanes 2 and 3). This suggests copper free cycloaddition can be used for biotin conjugation to azide functionalized substrate covalently attached to the A-PCP didomain displayed on the phage surface. We also found phages retained full infectivity after substrate loading with **4** and biotin conjugation with **5** without copper in the reaction.

We then tested if biotin-DIFO **5** can be used for specific selection of phages displaying A-PCP didomain of RifA with the loading of azide substrate **4**. A-PCP didomain displayed phages were activated by Ppant modification in the presence of Sfp and CoA followed by substrate loading with **4**. In parallel a control reaction was set up in which same number of phages displaying a single PCP domain of the NRPS module GrsA[33] was modified by Ppant and incubated with **4**. After PEG precipitation, the resuspended phage particles were reacted with **5**. Reaction mixtures were PEG precipitated again to remove unreacted **5** before they were added to a 96-well plate coated with streptavidin for binding the biotin conjugated phages. Plates were washed and *E. coli* cells were added to the plate for direct infection by phages bound to the plate. Phage infected cells were then titered and the results were shown in Fig. 3. Affinity selection with streptavidin binding gave more than a 10-fold increase in phage enrichment for phages displaying A-PCP didomain after substrate loading with **4** comparing to the control phages displaying just the PCP domain after incubating with the same substrate (Fig. 3, lanes 1 and 5). Also A-PCP displayed phages without Ppant activation did not give any phage enrichment over the control phages displaying the single PCP domain (Fig. 3, lanes 4 and 5). The enrichment of A-PCP displayed phages also relied on substrate loading with **4** and biotin conjugation with **5** (Fig. 3, lanes 2 and 3). These experiments demonstrate that copper free cycloaddition between an azide functionalized substrate loaded on phage displayed A-PCP and biotin-DIFO **5** can be used for the enrichment of A domain displayed phages based on catalytic turnover. Based on this method, substrate analogs of diverse structures with a common azide substitute can be used to identify matching mutants of the A domains that load nonnative substrates on the NRPS assembly lines. We are currently using this method to carry out selections on A domain libraries of RifA LM to identify A domain mutants with increased catalytic activities with **4**.

In summary we have developed a phage selection strategy to engineer the substrate specificities of the A domains of NRPS based on enzymatic loading of azide functionalized substrate analogs on

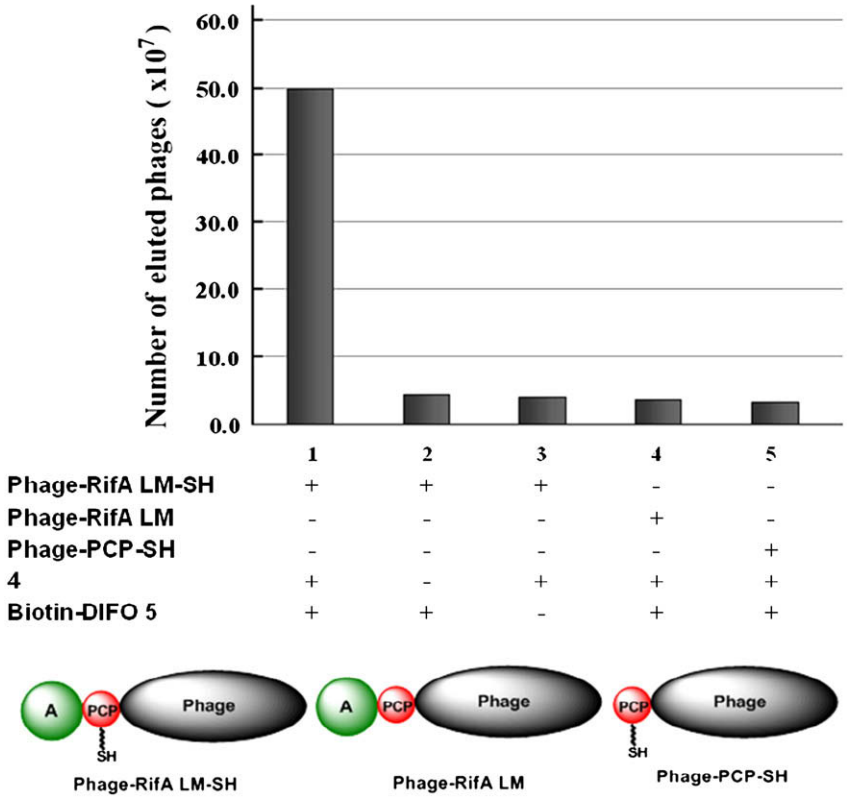

Figure 3. Phage titers for the enrichment of catalytic active RifA LM. Phages displaying the A-PCP didomain of RifA LM were loaded with substrate **4** and conjugated with biotin-DIFO **5**. Biotin-labeled phages were enriched by binding to a streptavidin plate and titered. Phages displaying the single PCP domain were used as a control.

the A-PCP didomain displayed on phage surface. We also applied copper free cycloaddition[31,32] for biotin attachment to the phage particles displaying catalytically active A-PCP didomains. We expect the same phage selection method can be used for displaying AT-ACP didomains from PKS modules on phage surface and selecting for substrate loading with azide functionalized acyl-CoA analogs. In this way, the substrate specificities of PKS and NRPS enzymatic assembly lines can be reprogramed for the incorporation of nonnative building block molecules into natural product biosynthesis. This method should be combined with existing methods of combinatorial biosynthesis[9] and precursor directed biosynthesis[14] to afford natural product analogs of diverse structures.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.08.085.

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