Chapter 1

mRNA display – a novel combinatorial peptide library

Abstract: mRNA display provides a strategy to make up to 10^{13} unique peptides that fuse with their own genes via a covalent linkage. *In vitro* selection using mRNA display libraries has been applied to ligand discovery and analysis. Compared to other *in vitro* selection methodologies, such as phage- and surface-display libraries, mRNA display libraries have several unique features, including the incorporation of non-native residues, the chemical derivatization of libraries, and the feasibility of mutagenic PCR and DNA recombination. These libraries should facilitate the discovery of novel ligands with functionalities beyond those provided by the 20 naturally occurring amino acids.

1.1 Introduction

1. Biological and chemical combinatorial peptide libraries

Combinatorial peptide libraries have been applied to select many functional peptides, such as receptor agonists[1] or antagonists[2], antibody epitopes[3,4], and enzyme inhibitors[5]. During the last decade, many powerful techniques to construct these libraries have been developed and provided various means for the isolation of peptides with desired chemical or biochemical properties. In general, combinatorial peptide libraries can be made from biological systems or chemical synthesis. Phage-display libraries[6,7], as well as ribosome-display[8], cell surface-display[9] and other biological libraries, share the common feature that each peptide is somehow associated with its encoding gene, allowing iterative cycles of enrichment and amplification. The identity of functional peptides from these libraries can be obtained directly by sequencing their tethered genes. Furthermore, some techniques, such as mRNA-display[10,11], can generate libraries containing up to 10^{13} unique peptides without any technical hurdles. Synthetic peptide libraries, by contrast, are unable to be amplified for repeating cycles of selection. The identification of active molecules also requires tedious de-convolution procedures[12] or sophisticated encoding-decoding schemes[13]. The complexity of synthetic libraries, for example, the one-bead-one-compound library, is usually only about 10^6. Nevertheless, the synthetic library can contain any unnatural amino acid residues, including fluorescent moieties, affinity tags and D-enantiomers[14,15]. The biological libraries, on the other hand, are limited to 20 naturally occurring amino acids. Thus, it would be a great advantage if unnatural amino acid residues could be introduced into a biologically generated library. The majority of this thesis covers several strategies to construct these libraries containing

unnatural residues. The application of these libraries to the isolation of high-affinity and high-specificity ligands is also discussed.

2. Common peptide libraries made from biological systems

Phage display, the most commonly used method for creating peptide biological libraries, is based on the expression of coat protein fusion on the surface of bacteriophage[6,16]. One of the coat proteins of the phage M13, termed gp13, is fused with N-terminal recombinant peptides encoded by synthetic random oligonucletides inserted at the 5'-end of the gene (gIII). The phages displaying various peptides on their surface can then be subjected to affinity selection. Those phages remaining bound to the immobilized targets after extensive washing are recovered and used to infect the bacteria for the next cycle of selection. Cell surface display libraries are very similar to phage display libraries in that peptides are expressed as a fusion to a membrane protein on the surface of bacteria or yeast.

In peptide-on-plasmid libraries, peptides are fused with a DNA-binding protein, which recognizes a specific region of plasmid containing the library sequences to form a stable non-covalent complex. During selection, the enriched library is amplified by liberating the plasmid for polymerase chain reaction.

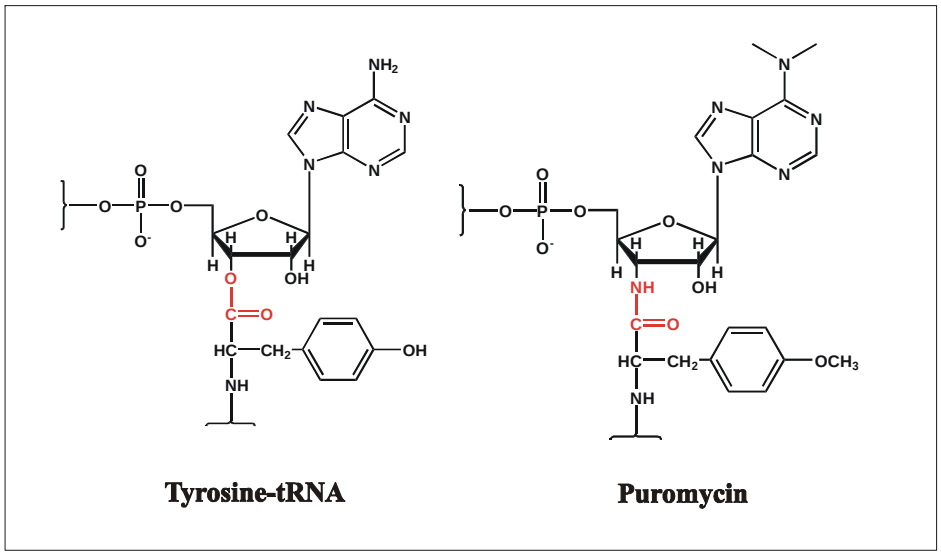
Ribosome display is another distinct technique for the construction of peptide libraries. The peptides are linked to their own messenger RNA via the formation of a tertiary mRNA-ribosome-peptide complex at low temperature and high magnesium concentrations.

Phage-, surface-, and plasmid-display libraries all require an *in vivo* step where the transformation efficiency limits the complexity of these libraries to approximately 10^9 members. Degradation of unfolded molecules, poor expression in the bacterial host, failure of processing to the phage surface, and failure to fold in the oxidizing periplasm, may further decrease the effective sequence representation. This *in vivo* step also makes it hard to manipulate the library sequences during selection through mutagenic PCR and DNA recombination. Ribosome display can achieve 10^{13} complexity easily. However, the mRNA-ribosome-peptide complex can only be maintained at very specific conditions, confining the system to applications that can be performed within a narrow range of thermal and chemical parameters.

The mRNA-display peptide library, originally described in 1997, has provided a novel approach for the selection of functional peptides and proteins with desired properties[10]. In this technique, messenger RNA templates are modified with a 3'-puromycin, a common translational inhibitor. Puromycin is a structural mimic of a 3'-tyrosinylated nucleotide, differing only in the replacement of unstable ester linkage between tyrosine and 3'-hydroxyl group by a stable amide bond (Figure 1.1). During translation, tyrosine-charged tRNA enters the ribosome, adds the tyrosine into the nascent peptide, and is released from the ribosome in the uncharged form. Puromycin, instead, stops the extension of the peptide, once it adds itself to the terminus of elongating peptide chain. Thus, translation of mRNA templates containing 3'-puromycin in rabbit retic lysate yields up to 10^{13} unique peptides fused with their encoding genes, serving as both templates and functional moieties[17] (Figure 1.2). The sequence information of the

peptides is stored in the covalently attached mRNA, and can be amplified for the recovery of the enriched library during affinity selection (Figure 1.3).

Figure 1.1 Chemical structure comparison between tyrosine-tRNA and puromycin.



The mRNA display library has certain advantages over other techniques in that 1) it is a fully *in vitro* strategy, and 2) the peptide is covalently linked to its encoding mRNA. Several strategies that are incompatible with other techniques, such as nonsense suppression, and chemical derivatization, have been demonstrated in mRNA-peptide fusion and considerably extend the application of mRNA display libraries. The optimization of fusion formation also makes it routine to produce more than $2x10^{13}$ fusions per milliliter of translation reaction, representing 10^4-fold increase in complexity over phage display, 10^6-fold over surface display, and 10^9 over colony screening approaches.

Figure 1.2 Process to prepare a mRNA display library. The mRNA template is modified with a 3'-puromycin molecule. During translation, puromycin can enter the ribosome and add to the nascent peptide chain to form a covalent link between mRNA template and its encoding peptide.

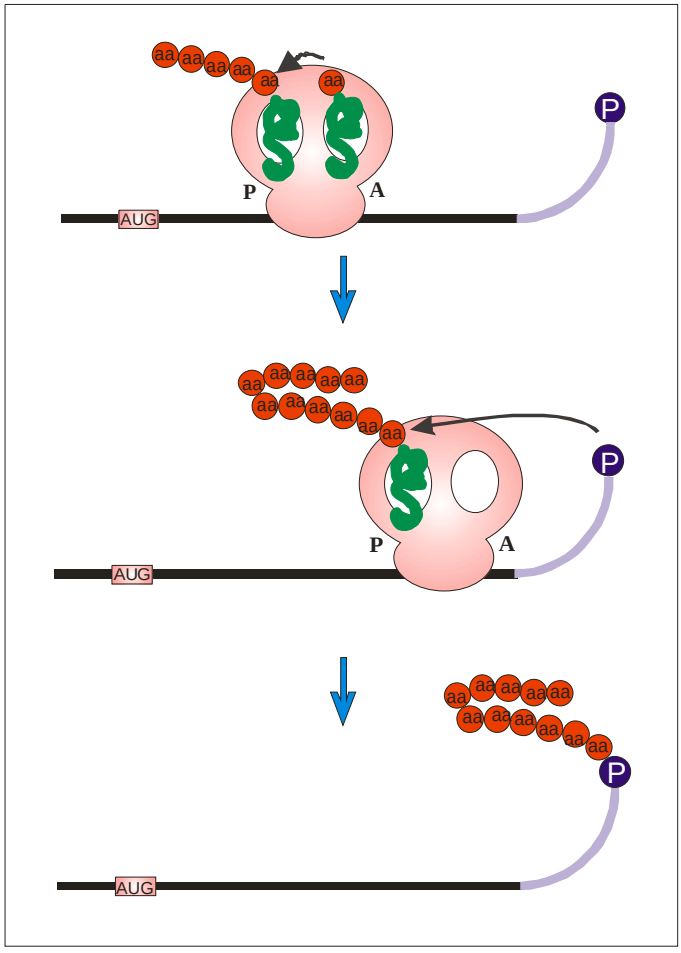
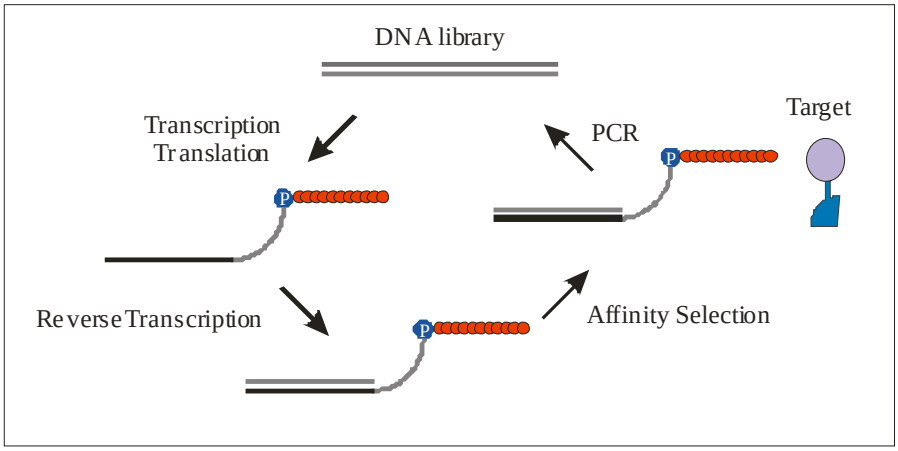


Figure 1.3 Affinity selection scheme with mRNA display library. The synthetic DNA library is transcribed to make mRNA, which is modified with a 3'-puromycin. The engineered mRNA template is translated to prepare mRNA-peptide fusion. After reverse transcription that generates cDNA·mRNA duplex, the fusion library is subjected to affinity selection against targets of interest. The enriched library is amplified by PCR for the next cycle of selection.



3. Applications of mRNA display libraries

mRNA display libraries have been used to identify novel ligands for many targets of interest, including ATP-binding aptamers[18], antibody epitopes[19], and RNA-binding peptides[20]. These results have demonstrated that diverse ligands can be selected from high-complexity peptide libraries.

a. ATP aptamers

An 80-residue peptide library was prepared from synthetic oligonucleotides by Keefe and Szostak to select ATP binding peptides[18]. The construction of libraries containing large numbers of random positions imposes several problems, including: 1)

the longer oligonucleotides are more prone to deletion and insertion during synthesis, and 2) the theoretical 3% rate of TAG stop codon incorporation at each random position (NNS) can dramatically reduce the fusion formation efficiency. To overcome these issues, Keefe adopted a prescreening procedure by selecting short fragments for the presence of N- and C-terminal epitope tags. This prescreen step considerably reduced the oligos containing stop codons and/or frame shifts. The full length library assembled from these readable fragments contains a higher percentage of members with the correct open reading frame.

A peptide with high affinity (K_d=100 nM) was identified after eighteen rounds of selection. In order to cover more sequence space, mutations were introduced into enriched library by error-prone PCR after eight cycles of selection. This peptide contains a conserved CXXC motif and binds an ATP molecule in a metal dependent manner, indicating a metal ion may help to maintain the peptide's overall structure.

b. Streptavidin aptamers

A binary patterned library was also designed in the Szostak laboratory. This library contained an 88-residue random region assembled from two 11-residue fragments with distinct hydrophobic and polar amino acid patterns[19]. Based on the patterning, the library was expected to form either amphipathic α-helices or β-strands. Surprisingly, after selection against immobilized streptavidin agarose, the enriched peptides showing high affinity with streptavidin were found to be frame-shifted, thus altering the designed patterning. The shifted frame was preferred in the selection due to the high percentage of the tripeptide (HPQ) in this frame. This HPQ tripeptide was first identified in a phage-display library selection as the minimum unit needed to bind with streptavidin. Several

peptides containing one or more HPQ motifs or the analogs, including HPA and LPQ motifs, were able to bind to streptavidin at sub-nanomolar level, representing more than 1000-fold improvement over strept-tag II peptide obtained from previous phage display experiments.

c. RNA binding peptides

The boxB RNA hairpin, the binding target of λ N protein, was used as a model system for RNA binding peptides selection[20,21]. In this selection, the initial library containing 10 random residues and representing about 10^{13} unique sequences has generated more than 100 distincts peptides that show strong affinity (K_d ~ 5 nM) against the boxB RNA hairpin. The lack of homology in these selected high-affinity RNA binding peptides indicate that the distinct peptides distributed throughout sequence space of the library possess the same binding characteristics. The research on how various peptides converge to the single wild-type RNA binding domain can shed light on further understanding of molecular evolution.

d. Epitope recovery

The mRNA display library was also applied to determine the sequences critical for antibody recognition. Baggio and coworkers prepared a 27 random residue library and used it for affinity selection against anti c-MYC monoclonal antibody 9E10[22]. The selection revealed a consensus core X-Q/E-X-L-I-S-E-X-X-L/M was required for effective recognition by antibody 9E10. This study demonstrated additional potential recognition sites for antibody 9E10 whose wild-type epitope is EQKLISEEDLN.

e. Self-assembling protein microarrays

Weng and coworkers demonstrated a novel application of mRNA-display library[23]. A DNA probe chip, whose oligo compositions are spatially addressable, can be converted to a protein chip. Each unique chimera molecule in mRNA display library can be directed to the proper coordinates on the DNA chip by a simple hybridization step. The resulting protein microarray preserves the functionality of the displayed proteins, keeps them in a uniform orientation, and has sub-attomole detection limits.

4. mRNA display library containing unnatural amino acid residues

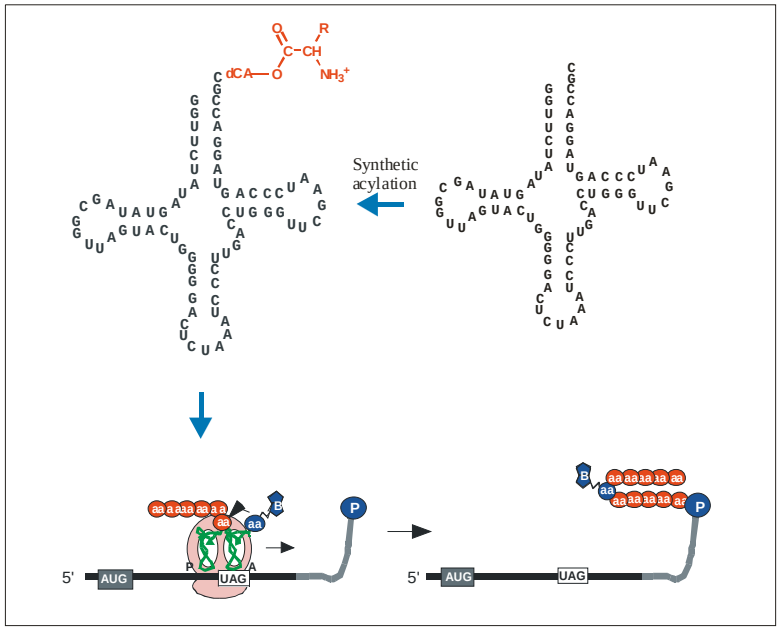
a. Incorporation of unnatural amino acid with UAG stop codon suppression

Techniques such as phage-display and cell surface display libraries demand an *in vivo* process, restricting the composition of these libraries to 20 naturally occurring amino acids. Recently, there are some advances in incorporating nonnative amino acids into proteins site specifically *in vivo*[24]. The unnatural amino acid is simply supplied as a nutrient to feed *E.Coli* and used by a modified tRNA synthetase to charge the UAG stop codon suppressor tRNA. When an mRNA template containing an in-frame UAG stop codon is translated, this nonnative residue is inserted into the elongating polypeptide at the position corresponding to the UAG stop. Even though several unnatural amino acids have been successfully incorporated by this method, the tRNA synthetase mutants can only take analogs of native residues. In addition, the incorporation of each unnatural amino acid requires tedious steps for selecting tRNA synthetase mutants that effectively charge tRNA with this amino acid. Thus, this technique is still hard to apply to combinatorial peptide libraries that require an *in vivo* step.

The mRNA display approach is a totally *in vitro* process, making it feasible to manipulate the library during the selection cycles. Techniques such as mutagenic PCR[25] and DNA shuffling[26] can be combined with mRNA display to enable the survey of more sequence space. More importantly, the *in vitro* UAG stop codon suppression enables us to insert unnatural amino acids into mRNA display library[27]. Incorporation of unnatural residues in protein via stop codon suppression was first demonstrated in the early 1990's and has been widely applied to incorporate fluorescent molecules, affinity tags, and spectroscopic probes into proteins at desired positions for purification and detection[28,29]. Unlike the *in vivo* UAG stop codon suppression developed recently, the problems associated with the charge of tRNA suppressor *in vivo* are avoided by making unnatural amino acid charged tRNA in a semi-synthetic manner. This semi-synthetic strategy starts with the protection of α-amine of amino acid with NVOC (4,5-dimethoxyl-2-nitrobenzyl) group and activation of carboxylic acid with chloroacetonitrile. The activated amino acid then reacts with a dinucleotide (pdCpA), and is subsequently coupled to the 3'-end of a transcribed 73-nucleotide tRNA by T4 RNA ligase to form intact charged suppressor tRNA. Prior to the translation, the NVOC protective group is cleaved from tRNA by UV irradiation. The addition of this semi-synthetic tRNA to the lysate in addition to a messenger RNA template containing an in-frame UAG stop codon can produce a peptide bearing the desired unnatural residue. It was demonstrated that the unnatural amino acids that are compatible with translational apparatus include not only various side chains, but also main chain structures. For example, 2 hydroxy acids can be inserted into proteins to form an acid-labile ester bond instead of normal backbone amide bond[30], and the insertion of N-methyl amino acids can produce a protease-resistant site[30]. Therefore, in

mRNA display, an engineered mRNA template containing both an in-frame stop UAG stop codon and 3'-puromycin should be able to form the mRNA-peptide fusion containing the unnatural amino acid, provided that the unnatural amino acid charged UAG suppressor is supplied (Figure 1.4). To demonstrate this strategy, biocytin-charged tRNA, a biotin derivative of lysine, was synthesized to insert the biocytin into mRNA display libraries. The incorporation of biocytin in the library was confirmed in two cycles of selection against streptavidin for enrichment of UAG stop codons in a library containing NNS random positions.

Figure 1.4 The incorporation of unnatural amino acids into mRNA-display library by amber stop codon suppression. The semi-synthetic charged tRNA can insert an unnatural amino acid in response to the UAG stop codon on the mRNA template.



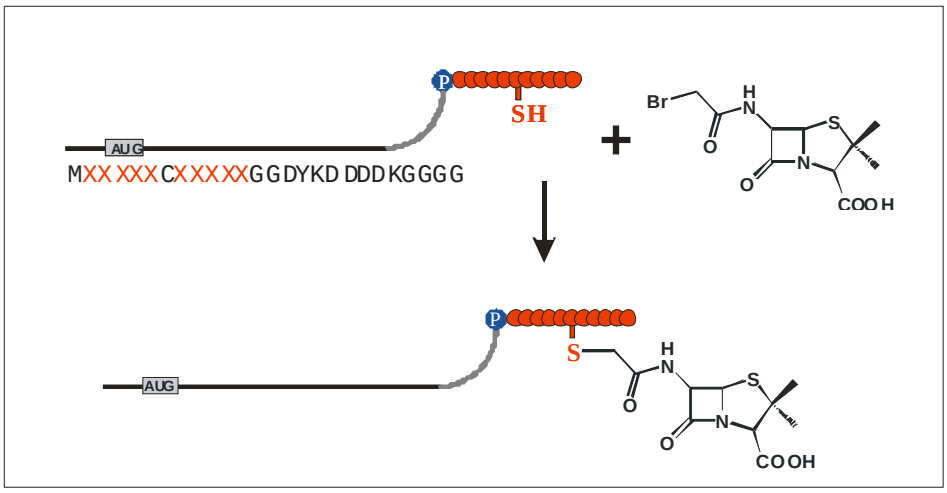
b. Incorporation of unnatural amino acid with chemical derivatization

One limitation of *in vitro* UAG stop codon suppression is that the unnatural residues incorporated must be compatible with the protein synthesis apparatus. Amino acids that 1) inhibit translation, 2) are reactive in retic lysate, and 3) are incompatible with ribosome can't be introduced into proteins. However, in mRNA display, peptides fuse with their own messenger RNA template through a covalent bond, making possible posttranslational chemical derivatization to incorporate an unnatural side chain.

Several specific chemical reactions have been extensively used for protein modification, including the coupling between the thio group of cysteine with maleimide or bromoacetyl functionality, and NHS with primary amine. The majority of protein labeling reagents available contain either cysteine reactive groups or lysine reactive groups due to their high-specificity and efficiency. Therefore, 6-bromoacetyl-penicillin was synthesized and incorporated into an mRNA display library containing a fixed cysteine[31] (Figure 1.5). This conjugation step is chemically orthogonal in that the bromoacetyl groups can not react with mRNA template and other 19 naturally occurring amino acids. The mRNA display library containing a pendant penicillin core was enriched for more potent inhibitors of penicillin binding protein 2a (PBP2a), a low-affinity penicillin binding protein that functions as a D,D-transpeptidase during bacterial cell wall synthesis of methicillin resistant *Staphylococcus aureus* (MRSA).

This chemical derivatization occurs after the translation so that any side chain, regardless its size, charge or chirality, can be introduced into fusion, considerably extending the chemical diversity of these libraries.

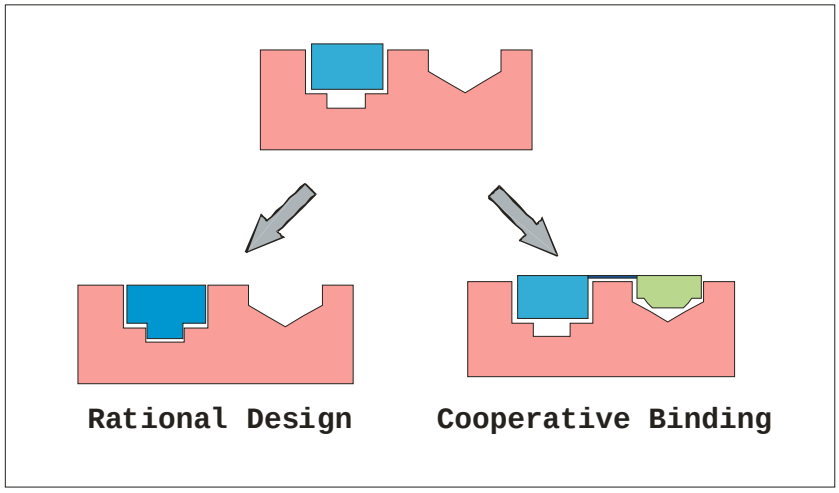
Figure 1.5 Chemical derivatization of the fixed cysteine residue with bromoacetyl-penicillin in an mRNA display library.



5. Applications of mRNA display library containing unnatural amino acids

Ligands with high affinity and high specificity are good drug candidates and excellent probes to explore the biological functions of target proteins. In pharmaceutical industries, low- or medium-affinity ligands can usually be obtained by screening a combinatorial library. To optimize the affinity of the lead compounds identified in the screen, a common approach is based on rational drug design, where the structure of lead compounds is modified to fit the active site of target proteins for better binding. Although extremely powerful, this strategy is dependent on the high-resolution structure of the target proteins, which is not always available. One interesting alternative is to utilize the cooperative binding of two molecules. The coupling of two modest-affinity non-competitive ligands can result in affinity that approaches the product of the affinities of two individual molecules, given the appropriate linker is used (Figure 1.6).

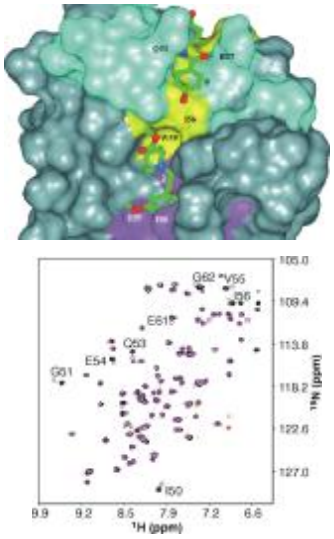
Figure 1.6 Two approaches for the optimization of ligands. One method to optimize a low-affinity ligand is rational design. Based on structural information, the ligand can be modified to fit the active site of target protein and improves its affinity. The other method is based on cooperative binding. The coupling of two modest-affinity non-competitive ligands can result in affinity that approaches the product of the affinities of two individual molecules.

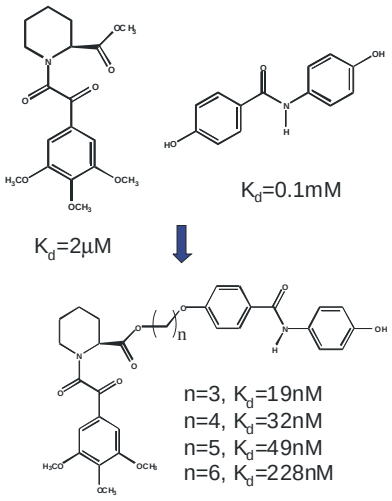


One of the examples that demonstrate this concept relied on structure-based linker design[32]. Shuker and coworkers used nuclear magnetic resonance (NMR) to optimize the linker between two weak binders of FKBP (FK506-binding protein), and were able to produce several chimeric molecules that bind to FKBP at least 100-fold better than either molecule alone (Figure 1.7). In most cases, however, only one weak ligand of the target protein is already known, thus making it necessary to search for both the second ligand and an appropriate linker. The mRNA display library containing the unnatural amino acid can be selected for these chimeric inhibitors where the unnatural amino acid serves

as the first binder. This affinity selection strategy searches for the second binder and the linker simultaneously, allowing the quick identification of high-affinity ligands.

Figure 1.7 High-affinity ligands of FKBP prepared from the cooperative binding of two weak non-competitive inhibitors of FKBP. NMR spectroscopy is used to optimize the connection between two inhibitors, whose K_d values are 2μm and 0.1mM, respectively. The resulting molecules have subnanomolar affinity.





Shuker S.B.,etal., *Science*, 274, 1531-1534 (1996)

1.2 Conclusion

mRNA display has provided a powerful tool for ligand discovery and interaction analysis. Many unique features are offered by this technique, including the option of mutagenic PCR and DNA recombination during selection, inclusion of nonnative residues, and chemical derivatization of libraries, create new opportunities in the field of molecule engineering. It also potentially enables the isolation of minimum artificial enzymes and the creation of wholly unnatural libraries with non-peptidic frameworks.

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Chapter 2

A novel strategy for *in vitro* selection of peptide-drug conjugates

Abstract: The chemical diversity of peptide and protein libraries generated from biological display systems is typically confined to the 20 naturally occurring amino acids. Here, we have developed a general strategy to introduce non-natural side chains into mRNA-display libraries via specific chemical derivatization. We constructed a mRNA-display library containing 3 x 10^{12} different peptides bearing a pendant penicillin moiety in a fixed position. *In vitro* selection using this hybrid peptide-drug library resulted in novel inhibitors of the *Staphylococcus aureus* penicillin-binding protein 2a (PBP2a). This strategy resulted in a penicillin-peptide conjugate that has at least 100-fold higher activity than the parent penicillin itself. Our approach provides a convenient way to enhance the efficacy of known drugs and facilitates the discovery of powerful new hybrid ligands with functionalities beyond those provided by the 20 naturally occurring residues.

Publication: Shuwei Li and Richard Roberts, *Chemistry and Biology*, in press

2.1 Introduction

1. Constructing mRNA display library containing unnatural amino acid

Combinatorial peptide libraries are rich reservoirs for discovery of novel ligands against therapeutically interesting targets, including receptor agonists[1] or antagonists[2], antibody epitope[3], and enzyme inhibitors[4]. Techniques such as phage display[5,6], ribosome display[7,8], and mRNA-display[9,10] can generate peptides that are physically associated with their own genes, making it feasible to identify molecules with desired properties through iterative cycles of enrichment and amplification. Because these libraries are made in biological systems, the chemical diversity is generally restricted to the 20 naturally occurring amino acids. By comparison, synthetic peptide libraries can contain numerous non-standard residues but typically represent much smaller sequence complexity than display approaches. Additionally, identification of active molecules in synthetic libraries often requires complex deconvolution[11] or sophisticated encoding strategies[12]. We have therefore been interested in developing approaches that allow non-natural residues to be incorporated into highly complex natural display libraries.

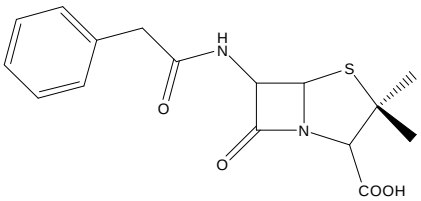
The mRNA display approach allows libraries containing more than 10^{13} independent peptides or proteins to be constructed entirely *in vitro*, providing the highest sequence diversity currently available with any method[13,14]. Additionally, because all steps in the mRNA display process are conducted *in vitro*, a variety of strategies are possible, such as *in vitro* mutagenesis[15], recombination[16], and nonsense suppression[17] that are not easily available to approaches such as phage display[6] or the yeast two-hybrid systems[18].

The introduction of unnatural side chains, such as affinity tags, spectroscopic probes, and analogs of the naturally occurring residues, into the desirable positions of proteins is a powerful tool for studying the properties of proteins, ligand-receptor interactions, and protein folding. Shultz and his coworker have pioneered to insert these unnatural residues site specifically into proteins by taking use of amber stop codon suppression[19]. A similar strategy used in our lab was able to insert biocytin, a derivative of lysine, into mRNA display library and reported in Chapter 3.

Although the translational apparatus in living organism can only take 20 naturally occurring residues, many proteins require various modifications, such as glycosylation, phosphorylation, and lipidation, for their normal functions *in vivo*. These modifications are usually performed in Golgi apparatus post-translationally and catalyzed by various enzymes. We have used this concept of post-translational modification to construct the mRNA display libraries containing unnatural side chains. Orthogonal chemical reactions between different functional groups, such as bromoacetyl or maleimide with cysteine, allow us to append desirable functional moieties to the unique residue in an mRNA display peptide library. This approach provides a convenient way to construct new molecular tools based on known pharmacophores, in that therapeutically useful small molecules may be presented adjacent to the chemical diversity present in a 10^{13}-member peptide or protein library. As a demonstration, a 11-mer peptide library containing a penicillin core was constructed to select more potent inhibitors of penicillin-binding protein 2a from a drug resistant pathogen, methicillin-resistant *Staphylococcus aureus* (MRSA).

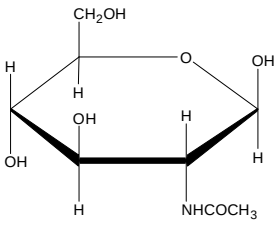
2. Penicillin antibiotics and their mechanism

Penicillin-type antibiotics are the most extensively used drugs to treat bacterial infections world wide. Since the discovery of the first penicillin drug more than 70 years ago, it has stimulated numerous research targeted to isolate or synthesize these compounds more efficiently, to understand the mechanism of their bactericidal action, and to obtain improved varieties overcoming drug resistances. Penicillin drugs can interfere with the synthesis of bacterial cell wall, the rigid barrier that keeps bacteria intact[20]. More precisely, penicillins are irreversible inhibitors of various penicillin binding proteins, enzymes catalyzing peptidoglycan cross-links.

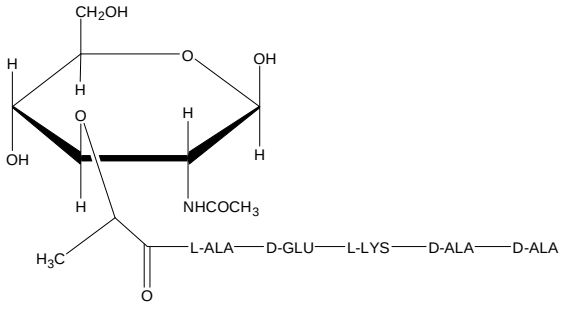


Structure of a typical penicillin drug benzyl-penicillin

Peptidoglycan is a huge mesh-like polymer in which linear strands of glycan, consisting of alternative N-acetylglucosamine (NAG) and N-acetylmuramic acid residues (NAM), join each other through short peptide cross-link. The length and composition of short peptides as well as the cross-link degree of peptidoglycan are species-dependent.



N-acetylglucosamine (NAG)



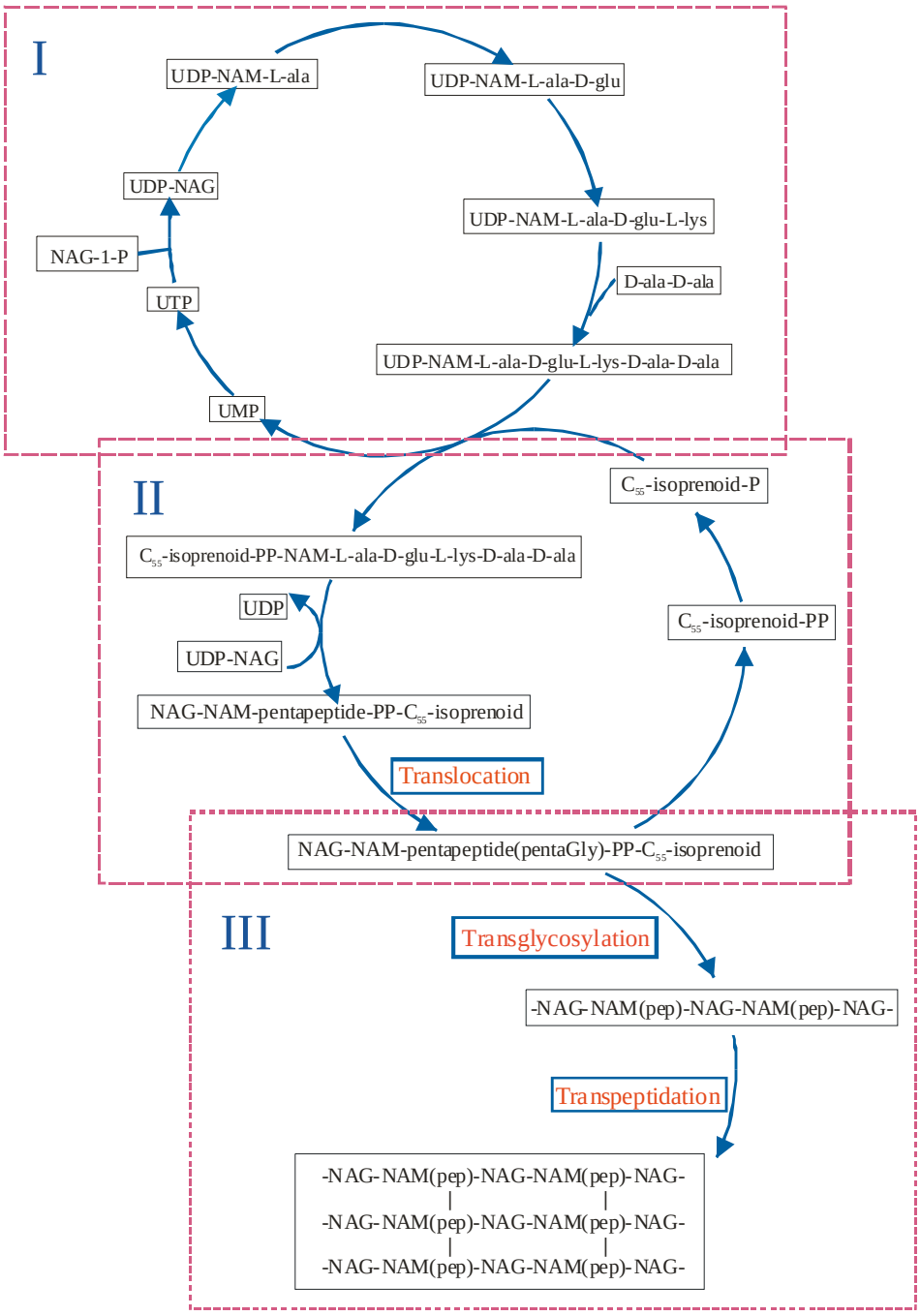
N-acetylmuramic acid-peptide (NAM)

The biosynthesis of bacterial cell wall is a multi-step process and involves many unique enzymes. Bacteria solve the problem of synthesizing a polymer larger than themselves in the extracellular space by (1) building activated precursors inside the cell;

(2) exporting them via a C55, membrane-soluble isoprenoid carrier; and (3) assembling the translocated pieces with the help of membrane-bound enzymes. For example, in *Staphylococcus aureus*, two precursor molecules, UDP-N-acetylglucosamine and UDP-N-acetylmuramyl-peptide, are produced in cytoplasm in the first stage. The peptide chain in UDP-N-acetylmuramyl-peptide ends with two consecutive D-alanines at C-terminus, where the last D-alanine is lost during the maturation of peptidoglycan. The second stage involves a series of reactions that further modify the precursor molecules. The UDP-N-acetylmuramyl-pentapeptide is phosphorylated and transferred to a monophosphorylated C55 isoprenoid alcohol. The UDP-N-acetylglucosamine then couples to NAM to form disaccharide-isoprenoid complex. A lysine residue in this molecule is then modified by sequential addition of five glycines, forming a branched peptide with exposed N-terminal amine. These building units eventually shuffle through the cytoplasmic membrane and remain anchored on the membrane via isoprenoid tail. The third and last stage consists of two distinct reactions, transglycosylation and transpeptidation. The transglycosylation reaction extends the length of peptidoglycan, while the transpeptidation closes the bridge between individual peptidoglycan chains (Figure 2.1).

There exist many types of antibiotics that target each stage of bacterial cell wall biosynthesis. Penicillins inhibit the last step, transpeptidation of peptidoglycan cross-link, which is catalyzed by membrane-bound D,D-transpeptidase, also called penicillin-binding proteins. These penicillin-binding proteins, including high molecular weight proteins up to 100 kD, as well as low molecular weight proteins about 30 kD, belong to the “active serine” enzyme family in that a serine is required for their normal function. This active serine can attack the amide bond between the C-terminal D-alanyl-D-alanine

Figure 2.1 Three-stage bacterial cell wall biosynthesis.



of peptidoglycan chain, forming an acyl-enzyme intermediate and releasing the last D-alanine concomitantly. The free N-terminal α-amine from the branch of another peptidoglycan chain then approaches the intermediate to form cross-link (Figure 2.2). Penicillins can permanently inactivate these D,D-transpeptidases by forming non-cleavable intermediate with them. Two factors contribute to their inhibitory capability: 1) they mimic the conformation of D-alanyl-D-alanine (Figure 2.3); 2) their tetra-member β-lactam ring is highly strained. Thus, the interruption of cell wall biosynthesis leads to the lysis of bacterial cells or other lethal phenomena in the presence of penicillins.

Numerous bacterial strains have developed drug resistance since the introduction of penicillin. One of primary reasons for resistance is caused by β-lactamases, a large family of enzymes that includes more than 200 unique members[21]. Both chromosome- and plasmid- encoded β-lactamases exist extensively in different drug-resistant strains. These enzymes can destroy β-lactam drugs before they reach the sites where cell wall biosynthesis occurs. TEM-1 β-lactamase, the major type of β-lactamases that is responsible for penicillin resistance in *E. Coli*, is a small 30 kD enzyme and belongs to serine hydrolase family. Similar to penicillin-binding proteins, the conserved serine-70 of the TEM-1 β-lactamase first forms ester bond with β-lactam core once a penicillin molecule occupies its active site. However, unlike the non-cleavable state in the penicillin-binding proteins, a water molecule activated by highly conserved glutamate-166 can sequentially attack the acyl-bond to release hydrolyzed product in TEM-1 β-lactamase (Figure 2.4). This reaction is so efficient that it can reach kinetic perfection for some suitable substrates, such as benzyl penicillin. The critical role of Glu-166 is demonstrated by a site-specific mutagenesis study. The Glu166Ala mutation of TEM-1

Figure 2.2 The mechanism of transpeptidation by D,D-transpeptidase (penicillin-binding proteins).

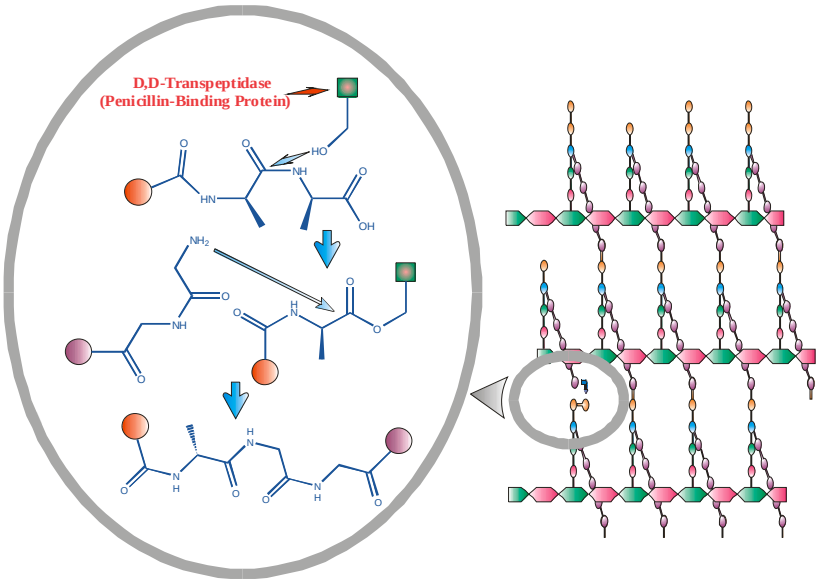


Figure 2.3 The conformation similarity between penicillin, cephalosporin and D-alanyl-D-alanine.

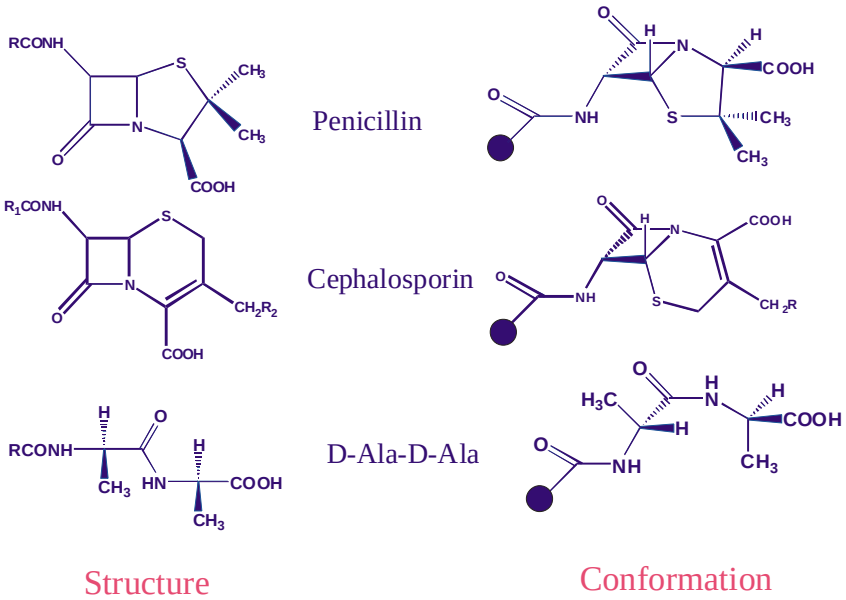
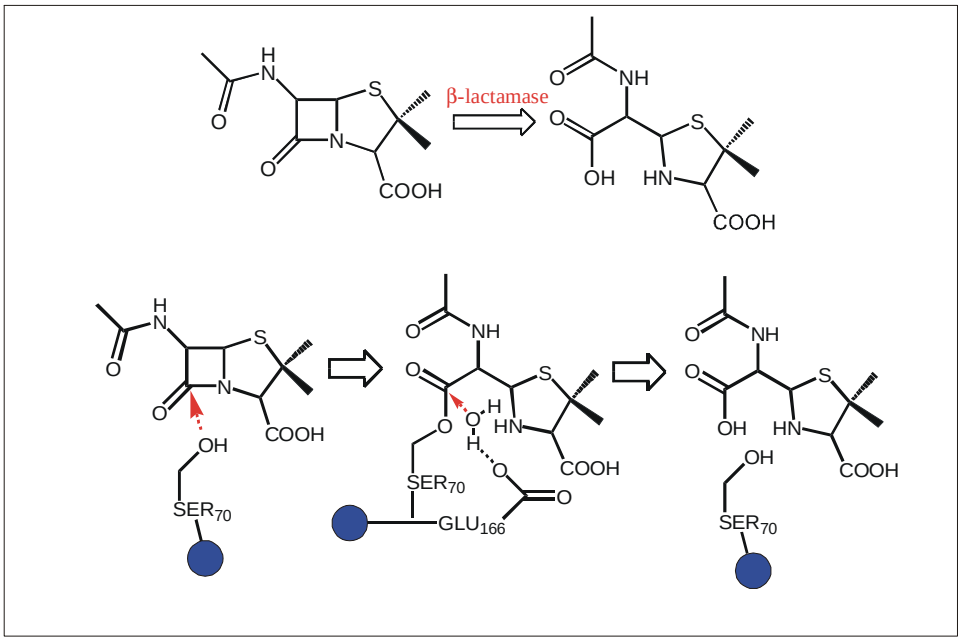


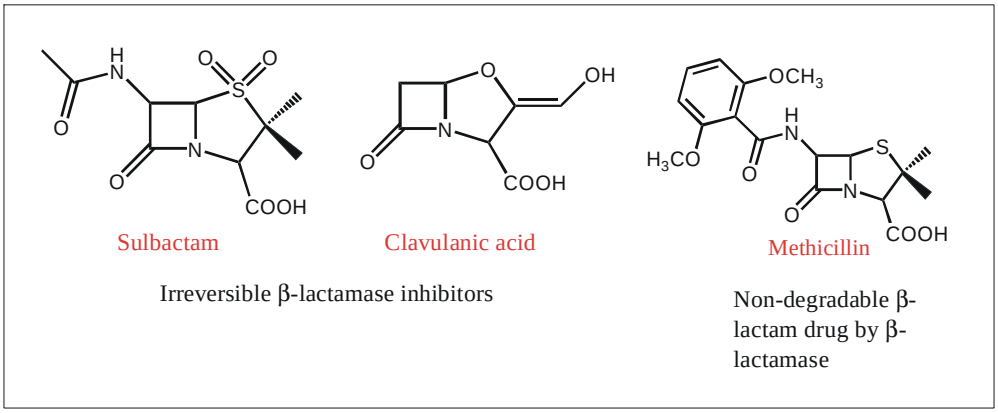
Figure 2.4 The mechanism of degradation of β-lactam compounds by β-lactamase.



β-lactamase can no longer catalyze the hydrolysis of β-lactams, leaving the enzyme trapped at acyl-intermediate[22]. To overcome problems imposed by β-lactamase, researchers have developed two intuitive approaches to counteract this type of bacterial drug resistance. One method is to co-administer the β-lactam antibiotics with irreversible inhibitors of β-lactamase, such as clauvlanic acid or sulbactam[23]. The synergetic effect resulting from this combination has proven to be very powerful in treatment of bacterial infections. The other method is to modify the β-lactam drugs themselves to render them non-degradable by β-lactamase. For example, the bulky group at the 6-position of β-

lactam in methicillin makes it inaccessible to the active site of most β-lactamases, thus preventing it from being destroyed (Figure 2.5).

Figure 2.5 Common clinically used drugs for penicillin-resistant bacterial infection caused by β-lactamase. These drugs include irreversible β-lactamase inhibitors such as sulbactam and clavulanic acid and non-degradable β-lactam molecule.



The second resistance mechanism evolved by bacteria is the emergence of low affinity penicillin-binding proteins. These proteins are still active when other high-affinity penicillin-binding proteins are inactived by high concentrations of β-lactam drugs. This imposes a more serious health threat and necessitates the development of more potent antibiotics[24]. Another resistance mechanism of bacteria, a less important process, is to efficiently pump out the penicillin drugs and only applies to some Gram-negative strains.

Penicillin-binding protein 2a (PBP2a) isolated from methicillin resistant *Staphylococcus aureus*, a 79 kD protein, is an important target for drug design. A soluble,

truncated form of PBP2a without the first 23 membrane-localization signal peptide was expressed and studied for its kinetic properties[25,26].

Previous kinetic studies have demonstrated that the reaction between penicillin binding protein (PBP) and a β-lactam molecule (Pen) follows a three-step mechanism, as listed by following equation.

$$\text{PBP+Pen} \underset{}{\overset{K_d}{\rightleftarrows}} \text{PBP•Pen} \xrightarrow{k_2} \text{PBP-Pen} \xrightarrow[H_2O]{k_3} \text{PBP+Penicilloate},$$

where K_d is the dissociation constant for the formation of the Michaelis complex (PBP·Pen), k_2 is the first-order rate constant for the acylation of forming covalently linked β-lactam/PBP adduct (PBP-Pen), and k_3 is the first-order rate constant for the deacylation of the PBP-Pen complex. In general, the k_3 is extremely slow (~ 10^{-5} S^{-1}) for penicillin binding proteins, making apparent second-order rate constant k_2/K_d the best representative of inhibitory potency of β-lactam drugs. In contrast, β-lactamase (Lac) interacts with β-lactam drugs in a similar fashion, but hydrolysis of Lac-Pen adduct is much more efficient. The kinetic parameters of PBP2a, a low affinity penicillin binding protein was also determined and found that its low affinity is due to a lower k_2 value. The k_2 value of PBP2a is 2-3 orders of magnitude less than that of high affinity penicillin binding proteins, while K_d is more or less in the same range. For example, benzyl penicillin binds weakly with R61 DD-carboxypeptidase (K_d=13 mM), a high affinity penicillin binding protein, comparable to PBP2a (K_d=13.3mM). However, the determined value of k_2=180 S^{-1} for R61 DD-carboxypeptidase is 3 orders of magnitude higher than that measured for PBP2a (0.22 S^{-1}). Recent structural determination of PBP2a-drug complex indicates the low k_2 value is caused by the requirement of

conformational change of β-lactam during the formation of acyl-PBP2a covalent adduct, which is not seen in other penicillin binding proteins. Nevertheless, to improve the inhibitory potency of β-lactam compounds against PBP2a, the β-lactam core structure can be modified to either increase k_2, decrease K_d, or both.

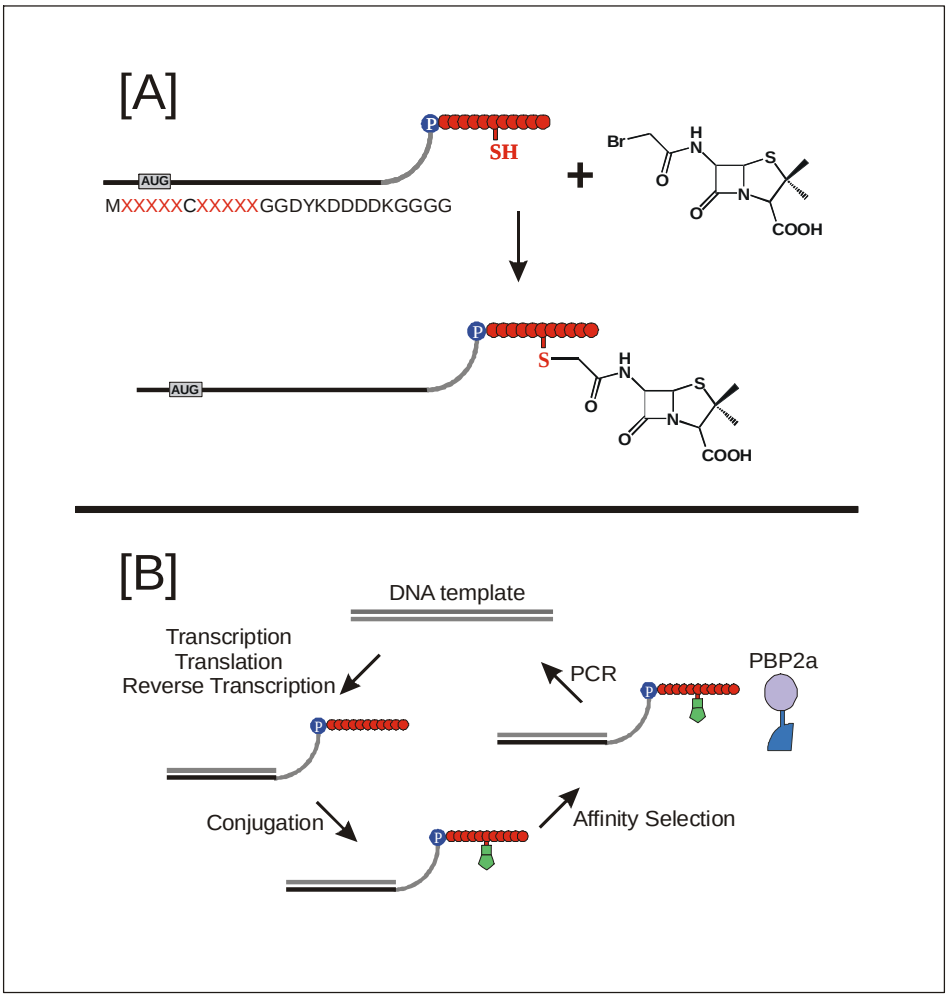
Another type of β-lactam compound, cephalosporin, differs from penicillins with a 6-member ring fusing with the core β-lactam (Figure 2.3). Recently, it was indicated that substituted cephalosporin ring has additional *van der Waals* contacts with the active site of PBP2a, providing higher affinity than the penicillin ring system[27]. However, the substitutions at 6-position of β-lactam in both series of drugs also exhibit influence to their affinity.

Previous studies to enhance the potency of PBP2a inhibitors have focused on the systematical modification of cephalosporin. The mRNA display library containing unnatural penicillin side chain is designed to provide an alternative strategy to sieve for more potent inhibitors of PBP2a.

3. The design of mRNA display library containing unnatural penicillin side chain

An mRNA peptide library where a fixed cysteine is flanked by 5 random residues on both sides was constructed. This fixed cysteine was modified with an unnatural β-lactam side chain through an orthogonal coupling reaction between the thio group and sodium 6-bromoacetylpencillanate (Figure 2.6 A). Such a "hybrid" library represented a huge collection of diverse β-lactam compounds with various peptides appended at the 6-position of the β-lactam ring. This library was used to select PBP2a inhibitors against immobilized PBP2a protein (Figure 2.6 B).

Figure 2.6 [A]. Construction of mRNA display libraries containing an unnatural penicillin side chain. The fixed cysteine residue is flanked by five randomized residues (in red) on both sides and react with sodium 6-bromoacetylpenicillanate to form fusion-penicillin conjugate. [B]. Selection cycle of the mRNA display library. DNA template generates the mRNA display library that conjugates with a penicillin ring through chemical derivatization. The resulting library is subject to affinity selection against immobilized PBP2a. Enriched fraction with improved properties are amplified by PCR.



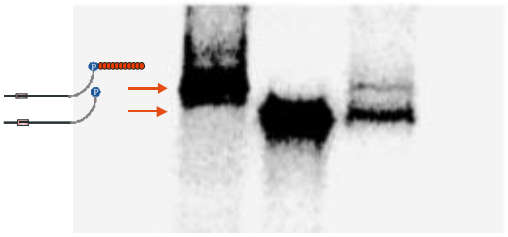
2.2 Results and discussion

1. Construction of mRNA display peptide library containing b-lactam side chain

The mRNA display peptide library was prepared by following the procedures described previously[28]. To examine the efficiency of mRNA fusion converted from mRNA template, a ^{32}P-labeled mRNA template was used in translation reaction. The result pointed out that the fusion formation efficiency was about 10-15%. It indicated that the entire library contained approximately 1.0-1.5×10^{13} unique peptides, representing near saturation coverage of peptide libraries with 10 randomized positions (20^{10}=10^{13}) (Figure 2.7 A). The freshly made libraries can be purified by thiopropyl gel to confirm that the constant cysteine residue was in the reduced state and ready for conjugation with sodium 6-bromoacetylpenicillanate (Figure 2.7 B).

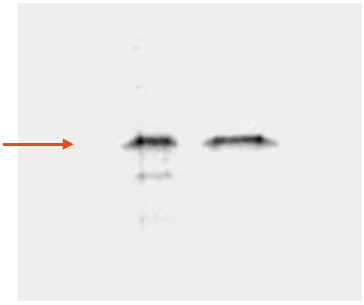
After coupling the library with sodium 6-bromoacetyl penicillanate, we needed to address two issues: (1) the fraction of the library bearing the drug and (2) whether any other functional groups on the peptide, RNA, or DNA were reactive with bromoacetyl moiety. The drug-modified mRNA display library is indistinguishable from unmodified libraries in standard PAGE experiments because the molecular weight difference between them is too small to be resolved. However, we were able to accurately quantitate the amount of drug-modified library using a hydrolysis-deficient mutant of the RTEM-1 β-lactamase (E166A). This 30 kD protein forms a stable covalent adduct with penicillin and penicillin derivatives at serine 70[22]. After library derivatization, we added the mutant β-lactamase (E166A) to the peptide fusion mixture and separated library members that could form a covalent bond with the mutant β-lactamase by electrophoresis (Figure 8A).

Figure 2.7 [A]. 10-20% formation efficiency of fusion from mRNA template. The first lane is the fusion labeled with ^{35}S-methionine, the second is the mRNA template labeled with ^{32}P-GTP, and the third lane is the fusion formation with ^{32}P-GTP labeled mRNA. [B]. Reductive state of cysteine in freshly prepared fusion. More 90% fusion can be purified by thiopropyl gel.



Labeling ^{35}S ^{32}P ^{32}P

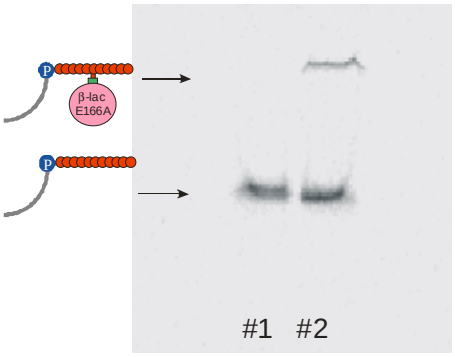
[A]



fusion fusion + thiopropyl gel

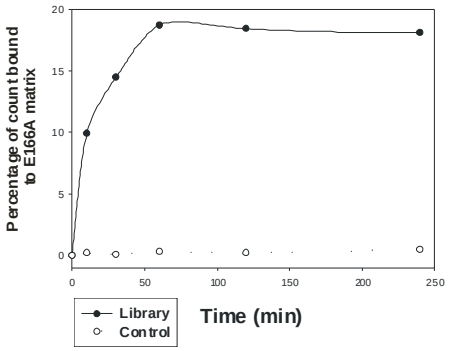
[B]

Figure 2.8 [A]. The conjugation of β-lactam with mRNA peptide fusion. Peptide fusion containing unnatural penicillin side chain can recruit β-lactamase E166 mutant. [B]. The orthogonal chemical reaction between bromoacetyl penicillanate and sulfurhydryl group in cysteine. Fusion containing cysteine can react with 6-bromoacety penicillin and bind to immobilized β-lactamase E166A mutant. The control without cysteine, instead, is unable to bind the E166A mutant after the conjugation step.



#1: fusion+E166A+RNase
#2: fusion+conjugation+E166A+RNase

[A]



Library: MXXXXXCXXXXXGGDYKDDDDKGGGG
Control: MHRNDESPTQYWGGDYKDDDDKGGGG

[B]

This analysis revealed that approximately 20% of the mRNA display library members contained a penicillin side chain and also demonstrated that a control mRNA display construct was unreactive with the bromoacetyl derivative of the drug (Figure 2.8 A and Figure 2.8 B). Therefore our starting library contained ~3 x 10^{12} different peptides bearing the drug as intended. Equally important, the bromoacetyl drug was chemically orthogonal with the functional groups on the template (hydroxyls, phosphates, ring nitrogens, exocyclic amines) and the non-cysteine amino acids (histidine, arginine, asparagine, glutamic and aspartic acid, serine, threonine, glutamine, tyrosine, lysine and tryptophan) as well as the N-terminal amine in the peptide.

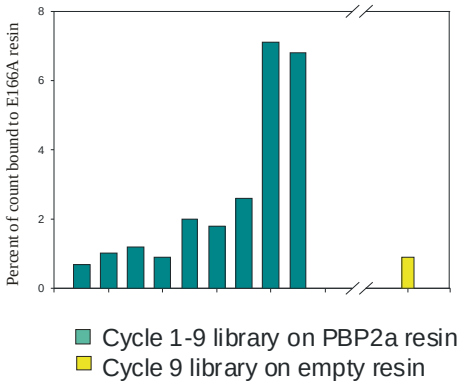
2. Selection against immobilized PBP2a

The penicillin-tagged fusion library was subjected to iterative cycles of selection for binding immobilized PBP2a (Figure 2.6 B; Figure 2.9 A). In each round, the library was eluted specifically by liberating the PBP2a from the matrix with DTT. As the selection progressed, we gradually increased the stringency by decreasing the incubation time of the library on the PBP2a matrix from 1 hour in the first-cycle to 10 minutes in the ninth-cycle. Penicillin forms a stable adduct with PBP2a, albeit very slowly under normal conditions. Our selection protocol thus selected for those members that could bind and react efficiently. After eight cycles of selection and amplification, the fraction of the library bound to the PBP2a matrix rose significantly above background. An additional ninth cycle resulted in no marked improvement. Under standard conditions, where libraries were incubated for 30 minutes, the fraction of library bound to the PBP2a increased from 0.4% of the first-cycle to 7% of the ninth-cycle library. This interaction

was specific for PBP2a, as little binding is seen when the round 9 library was tested with solid support alone (~0.6% binding; Figure 2.9 A).

We cloned and sequenced 15 individuals from the ninth-cycle library (Figure 2.9 B). The sequence analysis also revealed various mutations and deletions in the constant region of the templates. Cysteines appeared in randomized region of only two of the 15 clones, indicating that polyvalent binding did not predominate. We found no obvious consensus between these clones, implying many distinct peptides may be able to enhance the binding of β-lactam core molecule.

Figure 2.9 [A]. The percentage of ^{35}S-labeled libraries after every cycle bound to immobilized β-lactamase E166A mutant. The yellow bar is the percentage of the ninth library bound to the streptavidin beads without immobilized E166A. [B]. The sequences of 15 clones from the ninth library. Dot represents the identical base and hyphen represents deletion. The emerging cysteine highlight in red.



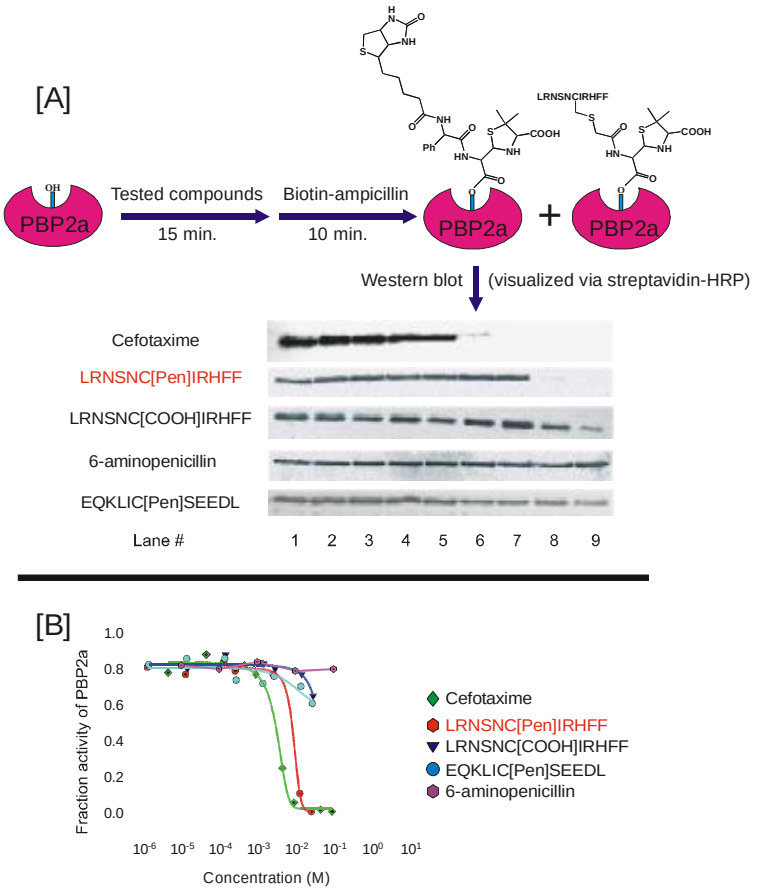

```
MXXXXXCXXXXXGGDYKDDDDKGGGG
.DRTPT.TFLSS............
.NFANV.VSQHI............
.ATKTD.FLSLV............
.NARWN.NSWLV............
.GRHTP.VSNLY............
.LRNSN.IRHFF.V...........(2)
.SPYRS.GLSAS.V..........
.RNNIT.RLLKR.V..........
.RACTT.SWPLS.V..........
.RHNLN.SAFWP.V..........
.SLSSI.LVPVA.ATTRTTTTRAAA
.ESHMV.RSTDV--..........
.SPAHC.PYLPF--..........
.SLAAH.AFPFL--..........
```

[A] **[B]**

3. Inhibition assay of enriched sequences

We synthesized the peptide LRNSNCIRHFF that occurred twice in the 15 clones, as well as its conjugate with sodium 6-bromoacetyl penicillanate (LRNSNC[Pen]IRHFF) to test their inhibition against PBP2a. To preclude the intermolecular disulfide bond formation between underivatized peptide, the free thiol group in the peptide (LRNSNCIRHFF) was blocked by reacting with bromoacetic acid. The resulting peptide conjugate (LRNSNC[COOH]IRHFF) and other control compounds were analyzed in a competition assay as described previously[29] (Figure 2.10 A). In this assay, various amounts of tested molecules are coincubated with fixed concentration of biotin-ampicillin, the competitive compound for certain time. After separation in standard SDS-PAGE, the percent of PBP2a containing covalently linked biotin moiety is determined by western blotting and chemiluminescent detection. Although it is not direct measurement of K_d, k_2 and other kinetic variables, this competitive assay can quickly compare the relative affinity of different β-lactam compounds against PBP2a. The relative IC_{50} obtained, defined as the concentration of a β-lactam molecule needed to inhibit half of the PBP2a in assay, is an indicator of apparent second-order rate constant k_2/K_d. We found that the relative IC_{50} of this peptide conjugate (LRNSNC[Pen]IRHFF) in our assay conditions was about 7 mM, making it a better inhibitor of PBP2a than either unconjugated peptide (LRNSNC[COOH]IRHFF) (IC_{50}>35 mM) or 6-aminopenicillanic acid (IC_{50}>500 mM) alone (Figure 2.10 B). A control peptide conjugate (EQKLIC[Pen]SEEDL) that did not appear in the final enriched library showed no such improvement (IC_{50}>35 mM). Our results indicated that substitution of the penicillin core with an appropriate 11-residue

Figure 2.10 [A]. The IC_{50} western blotting assay of molecules with biotin-ampicillin as a competitor. [B]. The IC_{50} curves derived from the western blotting.



oligopeptide greatly enhanced the efficacy of the drug against PBP2a. This enhancement likely occurs via interactions with the peptide and the protein near the active site, as the unmodified peptide also shows detectable activity. While the peptide conjugate (LRNSNC[Pen]IRHFF) is more than 100-fold improved compared to 6-APA, our selected molecule is also comparable to cefotaxime (IC_{50} =2.9 mM), a cephalosporin compound. Traditionally, the cephalosporin core structure has been the starting point for design of high affinity inhibitors of PBP2a because these compounds are usually more potent against PBP2a than penicillin systems. Recent structural work on PBP2a indicates the substituted cephalosporin ring has additional ***van der Waals*** contacts with the active site of PBP2a, providing higher affinity than the penicillin ring system[27]. Our results imply that the selected peptides attaching to 6-position of penicillin moiety are able to compensate the absence of these extra ***van der Waal***s interactions only seen in cephalosporin systems by contacting the surface area of PBP2a.

The modest IC_{50} (7 mM) that we observe does not necessarily imply that binding of our substrates is correspondingly weak. First, the absolute value of the IC_{50} depends on the conditions chosen for the assay, with longer conjugate-PBP2a incubation times producing correspondingly smaller IC_{50} values. Additionally, the rate constant for covalent attachment of penams to PBP2a (k_2) is quite slow, ranging from $k_2 = 0.22$ sec^{-1} for benzyl penicillin to $k_2 = 0.0083$ sec^{-1} for methicillin[30]. For comparison, the rate constant for formation of the acyl intermediate between penams and β-lactamase is ~2000 sec^{-1}, $10^4 – 10^6$-fold faster than PBP2a[31]. Covalent attachment of our compounds depends on the ratio of k_{-1}/k_2, as this value reflects how bound drug will partition

between dissociation and product formation. Values for k_1, the formation rate constant, are typically in excess of 10^8 $M^{-1}sec^{-1}$ for penams interacting with β-lactamase[31]. If we presume that $k_{-1} \sim k_2$ in order to give significant product formation, the predicted value of K_d would be nanomolar or below. If we conservatively estimate that k_1 is 10^6 $M^{-1}sec^{-1}$, typical for macromolecular association reactions, we predict values of K_d that are micromolar or below. Overall, the biochemical analysis indicates that the appended peptide facilitates the desired function of the drug by at least 100-fold. This observation is therefore consistent with our peptides adding approximately 3 kcal to the stabilization (k_1/k_{-1}), the reactivity (k_2), or some combination of the two to the parent penam drug.

2.3 Conclusion

The overall goal in this work was to extend the chemical diversity possible in mRNA display libraries through creation of functional drug-peptide conjugates. To that end, we have demonstrated that a penicillin side chain may be appended to mRNA display library in a chemically orthogonal fashion with reasonable synthetic efficiency. After the 9-round selection, all of the cloned sequences were in frame and contain a cysteine residue at the fixed position. These observations are consistent with the idea that the peptide-drug conjugate was formed and selected for interaction with the PBP2a, targeting our peptide library to the active site of the protein. Chemical synthesis of the peptide-drug conjugate confirms that this compound is in fact active against PBP2a, whereas neither the drug nor the peptide shows appreciable activity under concentrations that can be examined experimentally.

Perhaps the most compelling aspect of these experiments is their generality. There are numerous examples where drugs target specific sites in proteins[32,33]. The ability to generate peptide-drug conjugates enables the use of small molecules to direct display libraries to a particular face or site in a protein target of interest. Additionally, the covalent derivatization strategy we have used here enables library construction using compounds that would not be possible via *in vitro* nonsense suppression[17]. For example compounds that strongly inhibit translation such as kinase inhibitors or GTP analogs, or side chains that might be too large to fit in the exit tunnel of the ribosome (e.g, a heme) may not be efficiently inserted via nonsense suppression. We therefore anticipate that our approach should be broadly applicable for the development of novel tools to control biochemical processes.

2.4 Materials and methods

1. Preparation of sodium 6-bromoacetyl penicillanate

432 mg of 6-aminopenicillanic acid (Sigma, 6-APA, 2 mmol) and 500 mg sodium bicarbonate (Sigma, 4.5 mmol) were mixed in 4 ml water and 2 ml acetone with stirring for 10 minutes at 0ºC. 404 mg of bromoacetyl bromide (Aldrich, 2 mmol, 175 μl) was dissolved in 2 ml of acetone and added to the stirring solution. The reaction flask was wrapped with aluminum foil and kept on ice for 10 more minutes. The solution was removed from the ice bath and allowed to come to room temperature. The reaction was stirred for 1 hour and 5 ml of water was added to dissolve any residual white salt in the flask. The reaction was extracted twice with ether (4 ml), and then covered with 5 ml ethyl acetate. The reaction mixture was acidified with 40% phosphoric acid with stirring

at 0ºC. The ethyl acetate layer was removed, extracted (3X) with 5 ml distilled water and dried over anhydrous magnesium sulfate. The dried reaction mixture was combined with 300 µl n-butanol containing sodium 2-ethylhexanoate and stirred for 30 minutes. The precipitate was collected by filtration, washed with several portions of ethyl acetate, and air dried (395 mg).

To confirm the purity of product, 20 µl sodium 6-bromoacetyl penicillanate solution (10 mg/ml) was injected into HPLC and eluted with linear gradient (4 ml/min in a C_{18} reverse column, 0% buffer B to 100% buffer B in 20 minutes, buffer A: 95% water, 5% acetonitrile, 0.1% TFA; buffer B: 90% acetonitrile, 10% water, 0.1% TFA). The product was monitored at 254 nm and the retention time of sodium 6-bromoacetyl penicillanate is about 8.5 minutes.

The ESI-MS (electrospray ionization mass spectrum) confirms the product because it gives two equal peaks at 335 and 337 caused by two natural isotopes of element bromine (The expected mass peaks $[M-H]^-$ are 334.97 and 336.97) (Figure 2.11).

2. Preparation of mRNA display library containing unnatural penicillanate side chain

a. Construction of fusion template

Synthetic DNA template (sd7) were purified by preparative polyacrylamide gel electrophoresis. Polymerase chain reaction (PCR) of this template with two synthetic primers, sd2 and sd3, generates double stranded DNA. mRNA was produced by T7 runoff transcription of these templates in the presence of RNAsecure (Ambion) followed by size exclusion column purification (NAP25 column, Amersham Pharmacia Biotech). The flexible DNA linker containing puromycin, F30P, was synthesized using standard

Figure 2.11 Preparation of sodium 6-bromoacetyl penicillanate.

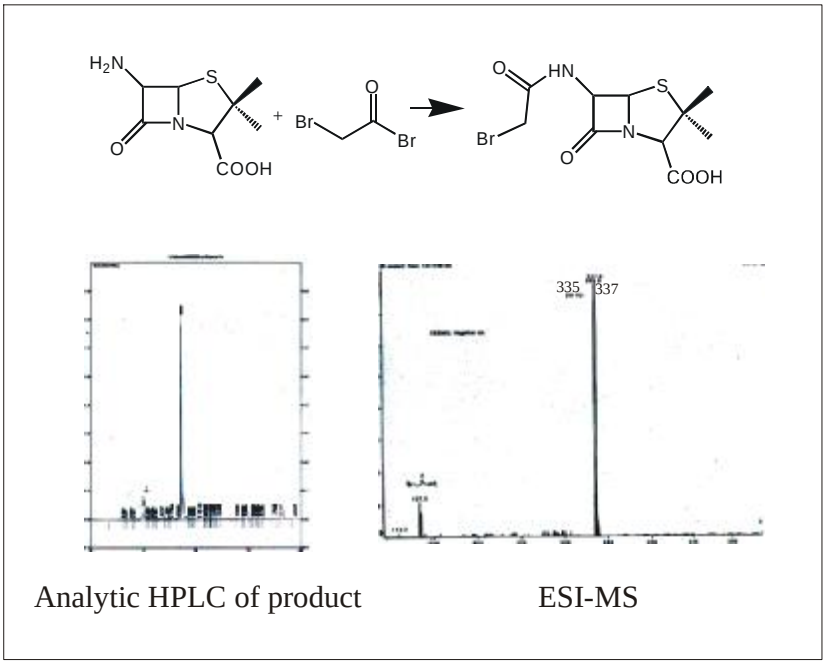


Figure 2.12 Synthesis of fluorescein-ampicillin.

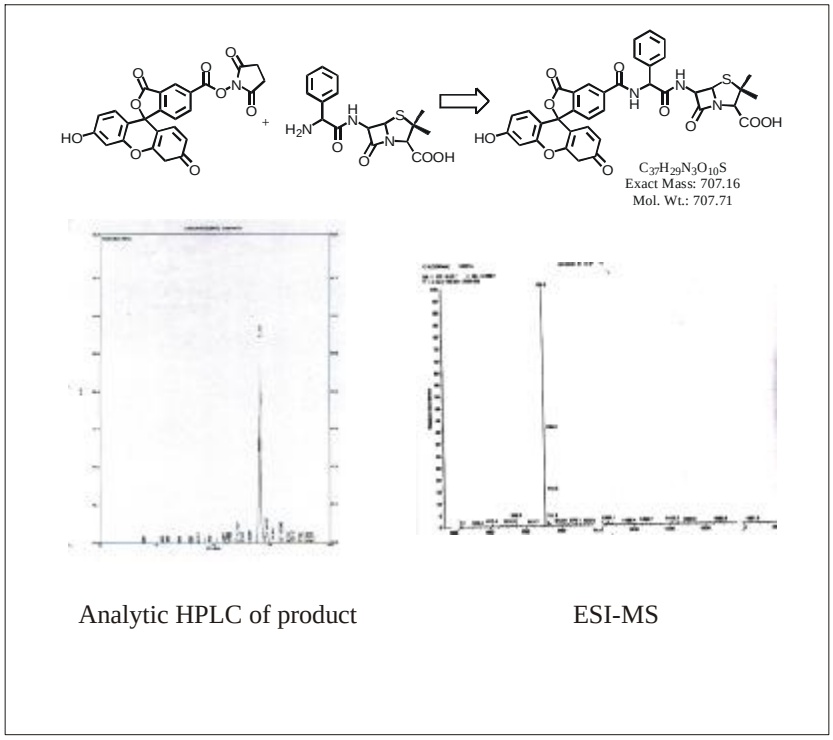
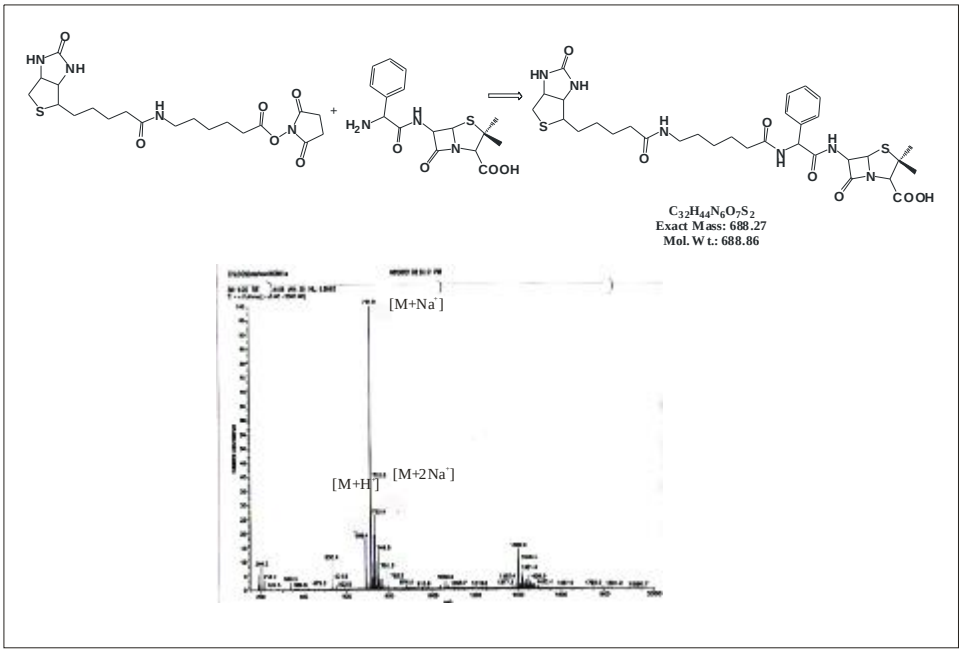


Figure 2.13 Synthesis of biotin-LC-ampicillin. The product is confirmed by mass spectrum.



chemistry. The oligonucleotide was chemically phosphorylated using phosphorylation reagent II (Glen Research) and purified by OPC cartridge. Ligation of pF30P to transcribed mRNA was done by mixing mRNA, pF30P, a splint oligo sd4 in a 1:0.5:1.2 ratio with 2 Units of T4 DNA ligase (New England Biolabs) per picomole of template mRNA. After ligation, the fusion template was gel-purified, electroeluted and desalted by ethanol precipitation. The precipitated mRNA was dissolved in water and further desalted with size exclusion column (Centri·Spin-20, Princeton Separation Inc.) and ready for *in vitro* translation.

b. Translation and fusion formation

The fusion template was translated in reticulocyte lysate (Novagen) using standard conditions (800 nM template) with the addition of ^{35}S-methionine as the labeling reagent. On completion of translation, fusion formation was stimulated by addition of $MgCl_2$ and KCl to 50 mM and 0.6 M, respectively, and incubated at -20ºC overnight.

c. Conjugation with sodium 6-bromoacetyl penicillanate

After purified with dT oligo cellulose, the mRNA display peptide library was used as template for reverse transcription with sd4 as the extension primer. The product was purified by phenol extraction and ethanol precipitation and dissolved in 300 µl reaction buffer (100 mM borate, pH=8.3), then, 8 µl tris(2-carboxyethyl) phosphin (TCEP, 20 mM, pH=8.0) was added and incubated at room temperature for 30 minutes. 1.1 mg sodium bromoacetyl penicillanate (10 mM in final solution) was dissolved in this solution and the mixture was shaken for 1 hour at dark. 30 µl DTT (2M) was added to stop the reaction by allowing reaction for extra 30 minutes. The fusion conjugate was ethanol precipitated and redissolved for selection.

d. Confirmation of conjugation

The fusion sample after treatment with sodium 6-bromoacetyl penicillanate were precipitated by ethanol with the addition of linear acryamide to 20 μg/ml and dissolved in 80 μl phosphate buffer (50 mM, pH=8.0). 20 μl β-lactamase E166A mutant (20 μg/ml) was added and the mixture was incubated at room temperature for 10 minutes. The β-lactamase was inactivated by heating the sample at 90ºC for 5 minutes. 2 μl RNase was added. After 10 minutes digestion, the sample was concentrated and directly loaded onto a 15% tricine-PAGE. The gel was dried and exposed on a phosphorus screen (Molecular Dynamics) for detection. A fusion sample without the conjugation was also processed as a control.

e. Specificity of conjugation reaction

An oligo template (sd23) that encodes all other 19 naturally occurring amino acid residues, but without cysteine was used to make fusion conjugation as described. The resulting fusion was incubated with immobilized β-lactamase E166A mutant on streptavidin agarose in phosphate buffer (50 mM, pH=8.0). The fraction of counts absorbed onto the beads were measured by scintillation counter after washing with washing buffer (50 mM phosphate buffer, pH=8.0, 100 mM NaCl, 0.1% Triton-X100) at various time points (0, 10, 30, 60, 120, 240 minutes). The normal fusion containing fixed cysteine was also test following the same procedure.

3. Preparation of immobilized penicillin binding protein 2a (PBP2a)

a. Purification of PBP2a

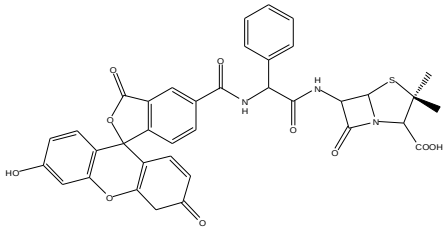
Plasmid bearing PBP2a gene was a gift from Professor Hiramatsu (Juntendo University, Tokyo, Japan). PBP2a gene was amplified by PCR with sd14 and sd16 as primers. The resulting DNA template was cut by restriction enzymes NcoI and SapI (New England Biolab) and ligated into PTXB3 plasmid (New England Biolab) to make plasmid PTXB3-PBP2a. The insertion was confirmed by restrict enzyme digestion assay. PTXB3-PBP2a was transformed into BL21(DE3) strain for expression. The induction with 1 mM IPTG was performed when the A_{600} of medium reached 0.6. The bacteria was shaken at 37ºC for 3 hours and harvested. Bacteria from 1 liter medium was collected and re-suspended in 80 ml column buffer (80 mM Hepes, pH=8.0; 500 mM NaCl; 1 mM EDTA). The solution was lysated with French Press and centrifuged for 20 minutes at 16000 RPM. The supernatant was collected and incubated with 10 ml chitin beads at 4ºC for 1 hour. The chitin column was washed with 100 ml column buffer. 20 ml cleavage buffer (80 mM Hepes, PH=8.0; 500 mM NaCl; 1 mM EDTA; 50 mM DTT) was added and the mixture was incubated at 4ºC for 24 hours. The elution then was collected and concentrated to 2 ml with centriprep YM-30 (Millipore). The concentrated PBP2a solution was dialyzed against phosphate buffer (50 mM, pH=8.0) and quantified by the Bradford assay.

b. Functional assay of PBP2a

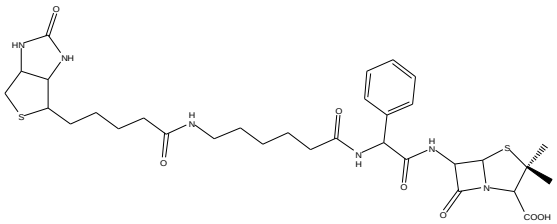
The purified PBP2a was tested for activity by reacting with fluorescein-ampicillin conjugate. After 30 minutes incubation, the mixture was directly loaded onto SDS polyacryamide gel. The activity of PBP2a was confirmed by the green fluorescence appearing at the position where PBP2a was (80 kD) under irradiation of a UV lamp (260 nm).

4. Preparation of fluorescein-ampicillin and biotin-ampicillin

Fluorescein-NHS (Pierce Chemical, 24 mg) or Biotin-LC-NHS (Pierce Chemical, 40 mg) were mixed with sodium ampicillanic acid (37 mg) in 1 ml cold phosphate buffer (50 mM, pH=8.0) and shaken at room temperature for 1 hour to prepare fluorescein-ampicillin (Figure 2.12) and biotin-ampicillin (Figure 2.13) respectively. The products were purified by HPLC directly at 254 nm wavelength using linear gradient (0% buffer B to 100% buffer B within 20 minutes, buffer A: 95% water, 5% acetonitrile, 0.1% TFA; buffer B: 90% acetonitrile, 10% water, 0.1% TFA) and confirmed by ESI-MS.



Fluorescein-ampicillin



biotin-LC-ampicillin

5. Selection against immobilized PBP2a

Purified PBP2a protein was reacted with biotin-SS-NHS (Pierce Chemical, 10 mM in 50 mM phosphate buffer pH=8.0) and dialyzed against phosphate buffer (50 mM pH=8.0). The resulting biotinylated PBP2a was incubated with streptavidin agarose gel and shaken for 30 minutes. After draining extra PBP2a solution and washed with phosphate buffer several times, the fusion conjugate was mixed with immobilized PBP2a agarose in 1 ml incubation buffer (50 mM phosphate, pH=8.0; 100 mM NaCl; 2 mM biotin) for variable time (1st-3rd cycles, 1 hour, 4th-7th cycles, 30 minutes, 8th-9th cycles, 10 minutes). The agarose then was washed with washing buffer (50 mM phosphate, pH=8.0; 100 mM NaCl; 0.1% Triton-X100) 6×500 µl. 500 µl cleavage buffer

(50 mM phosphate, pH=8.0; 100 mM NaCl; 100 mM DTT) was incubated with washed agarose for 1 hour. The supernatant was collected, desalted and concentrated with microcon Y-30 (30 kD MWCO). The concentrated solution was used for PCR amplification directly with sd2 and sd3 as primers.

6. Relative IC_{50} assay of selected peptide penicillanate conjugate

The selected peptides were synthesized in an ABI peptide synthesizer, then deprotected with TFA and purified by HPLC in a semi-preparative reverse column (4 ml/min, 0% buffer B to 30% buffer B in 30 minutes, to 100% buffer B in 5 minutes, to 0% buffer B in 1 minutes, buffer A: 95% water, 5% acetonitrile, 0.1% TFA; buffer B: 90% acetonitrile, 10% water, 0.1% TFA) by monitoring at 220 nm. The lyophilized peptides then was dissolved and reacted with sodium 6-bromoacetyl penicillanate at borate buffer (100 mM, pH=8.3). The conjugates of peptide with penicillanate were purified again by HPLC and lyophilized. The peptides were quantified by measuring the fluorescence emitted by their products with fluoscamine and calibrating with a standard curve if they do not contain tyrosine and tryptophan. Different concentration of conjugates were prepared by dissolving in reaction buffer* (50 mM phosphate, pH=8.0; 100 mM NaCl) and incubated with 2 μg PBP2a protein, respectively, at 37ºC for 15 minutes. 2 μg biotin-ampicillin was added immediately and incubated at 37ºC for 10 minutes and the reaction was stopped by heating the reaction mixture at 94ºC for 5 minutes. The samples were loaded into 12% SDS-PAGE for separation, and then transferred to a nitrocellulose membrane by blotting with sodium bicarbonate buffer (20% methanol; 0.32 g/l Na_2CO_3; 0.84 g/l $NaHCO_3$) at 400 mA for 3 hours. The

membrane was blocked with blocking solution (5% milk; 20 mM Tris, pH=8.0; 150 mM NaCl; 0.05% Tween-20) for 1 hour, then incubated with 10 ml TBS buffer (20 mM Tris, pH=8.0; 150 mM NaCl) containing 1 μg/ml streptavidin-HRP protein (Pierce Chemical) at room temperature for 1 hour. Washed the membrane with 50 ml TBS buffer 6 times at 5 minutes interval and covered it with femto-western blotting reagent (Pierce Chemical). The membrane was then put into alphaimager to collect chemiluminscent emission for 10-15 minutes.

* Some peptides containing multiple copies of acidic groups such as glutamic acid or aspartic acid may be hard to dissolve in the buffer. Diluted sodium hydroxide could be added to adjust the PH and solubilize peptides. For example, control peptide (EQKLICSEEDL) contains three glutamic acids and one aspartic acid, thus requiring the adjustment of PH when dissolved in buffer.

Oligo sequences:

Sd2:

5'-GGATTCTAATACGACTCACTATAGGGACAATTACTATTTACAACCACCATG

Sd3

5'-GCCGCCGCCGCCCTTGTCATCGTCGTCCTTGTAGTC

Sd4

5'-TTTTTTTTTTTTNGCCGCCGCCGCC
N: 75% T; 9.4%A, 9.4% G; 6.2% C

Sd7

5'-ACTATTTACAACCACCATGNNSNNSNNSNNSNNSTGCNNSNNSNNSNNSNN
SGGCGGCGACTACAAGGACGACGATGACAAGGGCGGCGGCGGC
N: 30% A, 30% C, 20% G, 20% T
S: 60% C, 40% G

Sd14

5’-CAATCCTCCGGCTCTTCCGCAGCCTTCATCTATATCGTATTTTTTA

Sd16

5’-GCGGGAATTCCCATGGCTTCAAAAGATAAAG

Sd23

5'-ACTATTTACAACCACCATGCACCGCAACGACGAGTCCCCCACCCAGTACT
GGGGCGGCGACTACAAGGACGACGACGACAAG

F30P

5’-d$A_{21}[C_9]_3$dACdCP;
C_9=triethylene glycol phosphate
P = puromycin

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Chapter 3

Incorporation of unnatural amino acid into mRNA display libraries by amber codon suppression

Abstract: The incorporation of unnatural amino acids into selectable, amplifiable peptide and protein libraries expands the chemical diversity of such libraries, thus considerably facilitating the process of obtaining ligands with improved properties (affinity, specificity, and function), particularly against therapeutically interesting targets. Biocytin, a biotin derivative of lysine, can be inserted into a mRNA-protein fusion molecule through amber stop codon suppression. The templates containing the codon corresponding to biocytin tRNA (a UAG stop codon) can be enriched by iterative cycles of selection against a streptavidin agarose matrix.

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3.1 Introduction

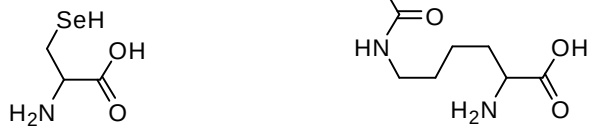
1. Amber stop codon suppression

Site-specific mutagenesis is an important approach for modifying the amino acid residues of proteins for structural and functional analysis. However, only 20 naturally occurring residues are convertible to one another in that the translational apparatus of living cells can only assemble these naturally occurring amino acids. The incorporation of unnatural amino acids into proteins is highly desired because it will add more available tools for the determination of various properties of proteins. For instance, the inclusion of a fluorescent probe in a protein can make this protein traceable for its role in a metabolite pathway or other cellular processes[1]. The introduction of an unnatural ketone group provides a specific site in a protein for selective post-translational modification[2,3]. The replacement of a key residue with an unnatural analog can also facilitate the determination of its functions[4].

The *in vivo* protein translational machinery is highly conserved in both prokaryotic and eukaryotic cells and consists of a large ribosomal complex and numerous proteins. Usually, the biosynthesis of a protein in a living cell involves initiation, elongation and termination. In the initiation stage, the ribosome scans through a messenger RNA (mRNA) to search for a starting point (AUG codon) in a suitable context. Once a start codon is found, a methionine-charged transfer RNA (tRNA) moves into the ribosome, recognizes this AUG codon, and serves as the first residue for protein synthesis. During the elongation stage, the ribosome processes along the mRNA template while various charged tRNA enter the ribosome to add more amino acids to the extended polypeptide chain dependent on the sequence of the mRNA template. Finally, when the

ribosome meets one of stop codons (UAG, UGA, or UAA), the nascent polypeptide chain leaves the ribosome, which itself also dissociates from the mRNA template. Other than mRNA and various tRNAs, this complex process also requires many accessory enzymes in every stage, such as initiation factors, elongation factors, and termination factors. The accuracy of protein translation from the genetic information contained in mRNA is controlled by aminoacyl-tRNA synthetases, enzymes that acylate tRNAs with different amino acids based on tRNA recognition properties. For example, isoleucine-tRNA synthetase is able to charge cognate tRNAs with isoleucine but not valine, whose structure differs from that of isoleucine by only a methylene group[5,6]. Such a high specificity provided by tRNA synthetases can keep the newly translated proteins from those catastrophic mutations due to the misacylation of tRNA. On the other hand, the editing function of tRNA synthetases also makes it extremely difficult to express proteins containing unnatural residues by simply supplying an unnatural amino acid to the growth medium.

In some rare cases, however, proteins newly translated *in vivo* do contain unnatural amino acids. For example, during the translation of formate dehydrogenase in *E. coli*, selenocysteine, which is not one of the twenty primary amino acids, is incorporated[7]. A more recent discovery is that the methylamine methyltransferases isolated from *M. barkeri* includes another unnatural residue, L-pyrrolysine[8]. The



selenocysteine

X=Me, NH_2, OH

pyrrolysine

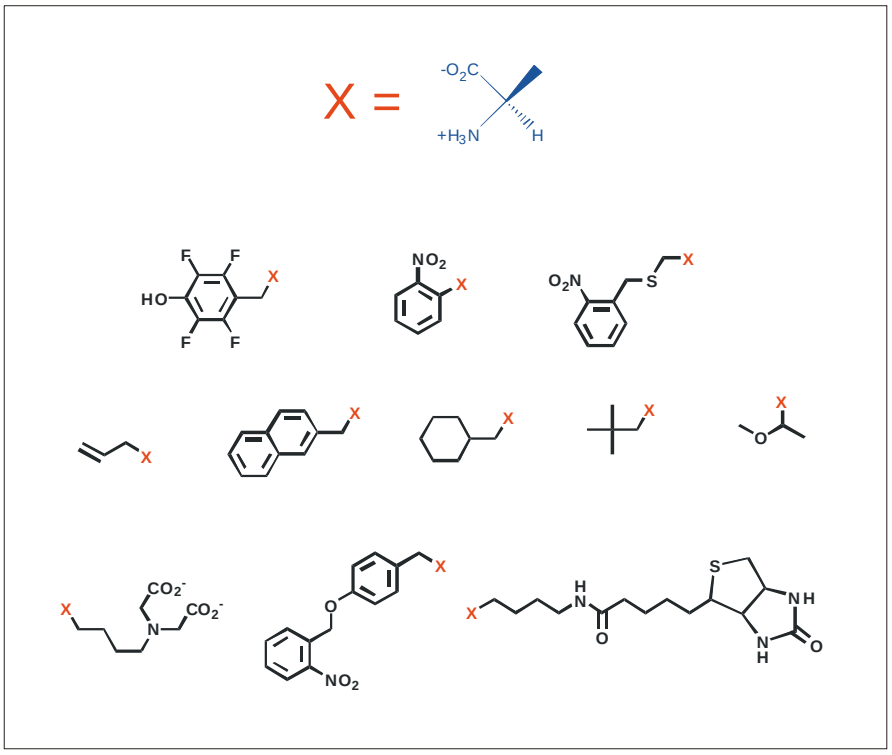
Two naturally inserted non-canonical amino acids

programming of unnatural amino acids into these proteins results from the existence of an in-frame UAG stop codon in their gene, a UAG codon tRNA suppressor, and a dedicated tRNA-synthetase that charges UAG stop codon suppressor with unnatural amino acids. Schultz and others have taken advantage of this observation and developed a semi-synthetic strategy to translate proteins carrying unnatural residues site-specifically that are compatible with ribosome[9].

The original semi-synthetic strategy was reported to translate proteins containing unnatural side chains in bacterial lysate[10]. A combination of chemical synthesis and run-off transcription was employed to prepare a misacylated-tRNA whose 3-base anticodon in the loop was replaced with an anti-UAG codon. Supplement of this semi-synthetic tRNA and mRNA template containing a UAG stop codon in the desirable position resulted in incorporation of the non-canonical residue. Many amino acids, including fluorescent or spectroscopic probes[1], affinity tags[11], and analogues for detailed mechanistic analysis[4], have been incorporated by this method (Figure 3.1). In addition to non-native side chains, various molecules different from standard amino acids can also be introduced. The acylation of tRNA suppressor with 2-hydroxyl acid, for instance, can replace the amide bond with a ester linkage that is acid-labile[12]. The efficient incorporation of N-methyl amino acids can generate proteins that are protease-resistant[12]. The semi-synthetic tRNAs were also injected into oocytes for *in vivo* expression of proteins containing non-natural residues[13].

Recently, Wang and his coworkers demonstrated that they were able to incorporate a O-methyl-L-tyrosine into Dihydrofolate Reductase (DHFR) in *E. coli.*, without the requirement of semi-synthetic tRNA[14]. Their approach starts with generating

Figure 3.1 Examples of unnatural amino acids incorporated into protein by amber stop codon suppression method[15].



an orthogonal tRNA and tRNA synthetase pair, which does not interact with other pairs existing in *E. coli*. Then the orthogonal synthetase was engineered so that it only charges the orthogonal tRNA with an unnatural amino acid. The orthogonal tRNA delivers the attached novel amino acid into proteins in response to a UAG codon inserted at any position of interest. Using this method, they have incorporated O-methyl-L-tyrosine into proteins with purity higher than 99 percent, which is close to the translation fidelity of natural amino acids. The application of this strategy in eukaryotic cells has been under investigation.

2. Combinatorial peptide libraries

Combinatorial peptide libraries have been extensively applied to select for novel peptides with desirable properties, including agonists and antagonists of receptors, inhibitors of enzymes, and hormones that control various metabolic pathways. Techniques such as phage-[16], ribosome-[17], and mRNA-display[18-20] can generate very large peptide and protein libraries and sieve them for functional molecules. These libraries share the same feature that the individual peptide is associated with its own encoding DNA or RNA template, which can be amplified easily by polymerase chain reaction (PCR) or the combination of reverse transcription and PCR (RT-PCR). This makes it possible to amplify the small fraction of peptides enriched with desirable properties for further improvement. For example, phage-display libraries, by far the most commonly used method for creating peptide libraries biologically, are based on the expression of coat proteins on the surface of bacteriophage, virus that infect bacteria. One of the coat proteins of the phage M13, termed gp13, is fused with N-terminal

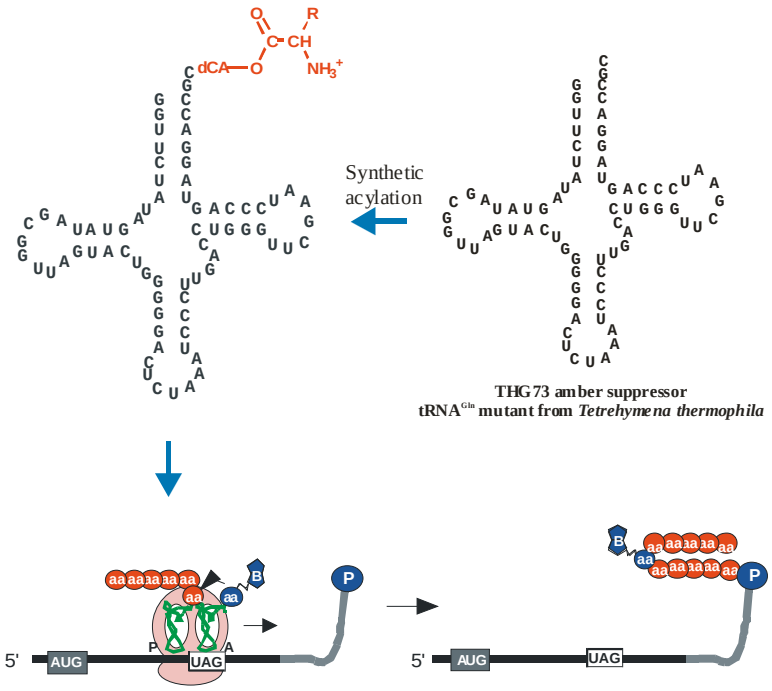
recombinant peptides that are encoded by synthetic randomized oligonucleotides inserted to the 5'-end of the gene (gIII). In ribosome-display libraries, the nascent peptides or proteins remain bound on mRNA through the non-covalent mRNA·ribosome·peptide complex, which is stabilized by a high concentration of magnesium ion and low temperature. The mRNA part of complex in enriched libraries against targets of interest contains the necessary genetic information for the iterative cycles. In mRNA-display libraries, translation extracts of rabbit reticulocyte lysate are used to generate combinatorial libraries of peptides and proteins covalently fused to their own mRNA via a 3' puromycin. These libraries are strictly monovalent and provide for the synthesis of more than 10^{13} independent peptide or protein sequences in a selectable format[21-25].

The chemical diversity that may be programmed into these libraries, however, is limited to the 20 naturally occurring amino acid side chains. Recently, there has been great interest in extending the unnatural strategy to systems where the novel residue may be selected for its function. Here, a combination of mRNA-display and UAG stop codon suppression has been developed to perform *in vitro* selection on mRNA display libraries containing the unnatural amino acid biocytin, a biotin derivative of lysine (Figure 3.2).

3.2 Results and discussion

First, mRNA-peptide fusions containing the unnatural residue biocytin were constructed. Biocytin, the biotin derivative of lysine, represented an excellent choice for the target residue as this has been inserted into proteins previously, and the biotin moiety could be readily used to select peptides that have incorporated this amino acid. The mRNA display libraries were prepared in the rabbit reticulocyte translation extract due to

Figure 3.2 Incorporation of unnatural amino acids into mRNA-display library by amber stop codon suppression. The semi-synthetic charged THG-73 tRNA can insert the unnatural amino acid in responsive to the UAG stop codon on mRNA template[13].



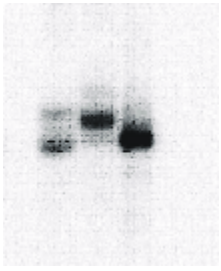
the excellent stability of the template in this media and the efficiency of fusion formation. The amber suppressor tRNA THG73 (a modified *Tetrehymena thermophila* Gln tRNA) was chosen to insert the unnatural residues by nonsense UAG suppression as this construct has high efficiency in eukaryotic translation systems (Figure 3.3).

Next, two templates were constructed to test insertion of the unnatural residue. The first template (Pep1) is a control containing all 20 amino acids, but no stop codon, while the second template (Pep2) contains a similar amino acid composition and a single UAG stop codon at the third position. For Pep2, fusion formation occurs only when the suppressor tRNA is added, consistent with incorporation of biocytin into the Pep2 mRNA-peptide fusion (Figure 3.4).

In order to demonstrate that this approach could be used to select peptides from libraries based on the function of the unnatural residue, the TTG codon that encoded the Trp residue in position 8 of the template pep1 was replaced with an NNS saturation cassette containing 32 possible codons encoding all 20 possible amino acids and the UAG stop (Lib1). Two rounds of *in vitro* selection were performed using streptavidin agarose as an affinity matrix.

Sequencing after one round of selection indicated that UAG stop codons were being enriched at both the randomized position and elsewhere in the open reading frame via point mutations. After a second round of selection vs. streptavidin agarose, nine clones were sequenced from the library. Eight out of nine (88%) contain a UAG stop codon at the randomized position or elsewhere, including two that contain a GAG to UAG transversion at position 5 (Figure 3.5).

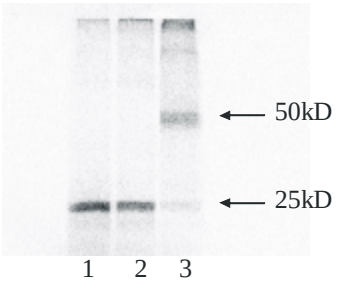
Figure 3.3 Ligation of truncated tRNA with dCA-biocytin gives functional amber codon suppressor. [A]. The ligated tRNA with pdCpA-biocytin is resolved from unligated tRNA. [B]. Supplement of biocytin-charged tRNA can suppress UAG stop codon to give full length 50 kD protein in rabbit retic lysate.



1 2 3

1: tRNA + T4 RNA ligase
2: tRNA + dCA-biocytin + T4 RNA ligase
3: ^{32}P labeled truncated tRNA (THG73)

[A]



1 2 3

1: nACHRα198 mRNA in lysate
2: nACHRα198+ truncated tRNA
3: nACHRα198 + suppressor

[B]

Figure 3.4 Dependence of fusion formation on addition of biocytin-charged THG73. [A]. Sequences of fusion templates tested. [B]. Gel showing formation of fusion products, labeled with 35S-methionine, in the presence (+) or absence (-) of biocytin-charged THG73. Fusion formation on the stop codon-containing template (Pep2) occurs only in the presence of the suppressor tRNA.

[A]

```
Sd29: MG.LDYKDEDKRQEIHLADLCKPFWVYTSGGGG
Sd25: MGRQEIHWANDLCKPFWVYTSGGGG
```

[B]

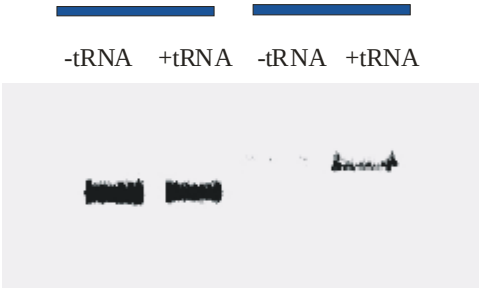


Figure 3.5 Sequences present in Lib1 before and after selection vs. streptavidin agarose. Amino acids sequence in 3-letter format of Lib1 before selection is shown in the second line and its DNA sequence is shown in the third line. Xxx represents all 20 amino acid residues plus UAG stop codon. N is equal amount of all 4 nucleotides and S is 50% G plus 50% C in that position. The sequences in NNS saturation region are highlighted in green and the emerging UAG stop codons are highlighted in red. The sequences same as the original template are labeled with dash (-).

```
 1          5             10             15             20             25
MetGlyArgGlnGluIleHisXxxAlaAsnAspLeuCysLysProPheTrpValTyrThrSerGlyGlyGlyGly
ATGGGCCGCCAGGAGATCCACNNSGCCAACGACCTGTGCAAGCCCTTCTGGGTGTACACCTCCGGCGGCGGCGGC
                  Sequences of 9 clones before selection
--------------------AAG----------------------------------------------------
--------------------AGC----------------------------------------------------
--------------------CCG----------------------------------------------------
--------------------TGC----------------------------------------------------
--------------------GTC----------------------------------------------------
--------------------TGG----------------------------------------------------
--------------------CAG----------------------------------------------------
--------------------AGC----------------------------------------------------
--------------------ATC----------------------------------------------------
              Sequences of 9 clones after one round selection
--------------------TAG----------------------------------------------------
--------------------TAG----------------------------------------------------
--------------------TTG----------------------------------------------------
--------------------AAG----------------------------------------------------
--------------------GAC----------------------------------------------------
--------------------GTG----------------------------------------------------
--------------------CAG----------------------------------------------------
--------------------AGG----------------------------------------------------
------------TAG------CAG----------------------------------------------------
             Sequences of 9 clones after two rounds selection
--------------------TAG----------------------------------------------------
--------------------TAG----------------------------------------------------
--------------------TAG----------------------------------------------------
--------------------TAG----------------------------------------------------
------------TAG------CAG----------------------------------------------------
------------TAG------CAG----------------------------------------------------
--------------------TAG----------------------------------------------------
--------------------TAG----------------------------------------------------
--------------------GAG----------------------------------------------------
```

3.3 Conclusion

This experiment represents the first combination of an *in vitro* selection experiment and nonsense suppression. It now allows selectable peptide and protein libraries to be constructed containing any non-natural amino acid that is compatible with the translation apparatus. These libraries should facilitate the discovery of novel ligands with functionalities beyond those provided by the 20 naturally occurring residues. For example, peptides containing N-substituted amino acids are protease resistant[26] and can show enhanced affinity for natural protein interaction modules such as SH3 domains[27]. This approach should also aid physical organic dissection of protein interfaces, particularly where molecular interactions display context dependence. Additionally, this approach provides a convenient way to construct new molecular tools based on known pharmacophores, in that therapeutically useful small molecules may be presented adjacent to the chemical diversity present in a 10^{13}-member peptide or protein library. Finally, translation systems that allow insertion of two or more unnatural amino acids[28] now provide the intriguing possibility for construction of wholly unnatural libraries in a selectable mRNA display format.

3.4 Materials and methods

1. Synthesis of biocytin-tRNA suppressor (Figure 3.6)

a. Synthesis of NVOC-biocytin cyanomethyl ester

Biocytin (100 mg, 0.26 mmol, Molecular Probes) and sodium carbonate (56 mg, 0.54 mmol) were dissolved in a mixture of water (15 ml) and THF (10 ml). A solution of

6-nitroveratryloxycarbonyl chloride (NVOC-Cl) (74 mg, 0.26 mmol, Sigma) in 10 ml THF was added slowly. After 3 hours, solvents were removed *in vacuo*. Then, 3 ml of dry DMF and 3 ml of chloroacetonitrile, as well as 800 μl triethylamine was added into remaining residues. After overnight stirring, solvents were removed *in vacuo* and the remaining solid was purified by flash chromatography (silica gel, 10% MeOH in CH_2Cl_2).

b. Preparation of biocytin-dpCpA

Tetrabutylammonium salt of dinucleotide (dpCpA) was a gift from Prof. Dennis Dougherty's group (Division of Chemistry and Chemical Engineering, California Institute of Technology). At room temperature, 10 mg dpCpA (8.3 μmol) and 16 mg NVOC-biocytin cyanomethyl ester (25 μmol) was mixed in dry DMF under argon. 20 μl ammonium acetate (25 mM, pH 4.5) was added to quench the reaction after 1 hour stirring. The crude product was purified by reversed phase semi-preparative HPLC using a gradient from 25 mM NH_4OAc (pH 4.5) to CH_3CN. The appropriate fractions were combined and lyophilized. The resulting solid was redissolved in 10 mM acetic acid/CH_3CN and lyophilized to give 3 mg biocytin-dpCpA as a pale yellow solid. The product was confirmed by mass spectrum as it gives a peak ($[M+H]^+$) at MW=1230.4 (Figure 3.7).

c. In vitro transcription of tRNA

THG73 tRNA was synthesized *in vitro* from FokI linearized plasmid harboring THG73 tRNA gene (gift from Prof. Dennis Dougherty's group) using T7 MEGAshortscript kit (Ambion). The product was purified by polyacrylamide gel electrophoresis and dissolved in water.

d. Ligation of biocytin to THG73 tRNA

The mixture of THG73 tRNA (25 μg in 10 μl water) and HEPES (20 μl, 10 mM, pH 7.5) was heated at 94ºC for 3 minutes and cooled down to 37ºC slowly. 8 μl biocytin-dpCpA (3 mM in DMSO), 32 μl 2.5X reaction buffer (25 μl 400 mM pH 7.5 HEPES; 10 μl 100 mM DTT; 25 μl 200 mM $MgCl_2$; 3.75 μl 10 mM ATP; 10 μl 5 mg/ml BSA; 26.25 μl water; 1 μl RNasin (Promega)), 5 μl water, as well as 5 μl T4 RNA ligase (New England Biolabs) was added. After 1 hour incubation at 37ºC, the reaction mixture was extracted once with an equal volume of phenol (saturated with 300 mM sodium acetate, pH 5.0):$CHCl_3$:isoamyl alcohol (25:24:1), then precipitated with 3 volume of cold ethanol at -20ºC. The precipitate was washed with cold 70% (v/v) ethanol, dried under vacuum, and resuspended in 5 μl 1mM sodium acetate, pH 5.0. The amount of biocytin-tRNA was quantified by measuring A_{260} and the concentration was adjusted to 1 μg/μl with 1 mM sodium acetate (pH 5.0). Prior to the suppression reaction, the biocytin-tRNA solution was deprotected by xenon lamp equipped with a 315 nm cut-off filter for 5 minutes.

2. General procedure to make mRNA-peptide fusions

a. Construction of fusion template

Synthetic DNA templates including Pep1, Pep2, and Lib1 were purified by preparative polyacrylamide gel electrophoresis. Polymerase chain reaction (PCR) of these templates with two synthetic primers, sd2 and sd26, generates double stranded DNA. mRNA was produced by T7 runoff transcription of these templates in the presence

of RNAsecure (Ambion) followed by size exclusion column purification (NAP25 column, Amersham Pharmacia Biotech). The flexible DNA linker containing puromycin, F30P (5'-$dA_{21}[C_9]_3$dACdCP; C_9=triethylene glycol phosphate, Glen Research; P = CPG-puromycin, Glen Research), was synthesized using standard chemistry. The oligonucleotide was chemically phosphorylated using phosphorylation reagent II (Glen Research) and purified by OPC cartridge. Ligation of pF30P to transcribed mRNA was done by mixing mRNA, pF30P, a splint in a 1:0.5:1.2 ratio with 2 Units of T4 DNA ligase (New England Biolabs) per picomole of template mRNA. After ligation, the fusion template was gel-purified, electroeluted and desalted by ethanol precipitation.

b. Translation and fusion formation

The fusion template was translated in reticulocyte lysate (Novagen) using standard conditions (800 nM template) with the addition of ^{35}S-methionine as the labeling reagent. In the case of templates containing UAG stop codon, 2 μg of deprotected biocytin-tRNA suppressor was also added. On completion of translation, fusion formation was stimulated by addition of $MgCl_2$ and KCl to 50 mM and 0.6 M, respectively, and incubated at -20ºC overnight. The resulting ^{35}S-labeled mRNA peptide fusions were directly loaded to 15% tricine SDS-PAGE for separation. After running, the gel was dried and exposed to phosphor screen (Molecular Dynamics) for several hours. The phosphor screen was then scanned to give image shown on Figure 3.4 B.

3. Enrichment of UAG stop codon by selection against streptavidin-agarose matrix

a. Template-base (dT) purification

To isolate fusion, the lysate was diluted in binding buffer (1M NaCl, 20 mM Tris pH 8.0, 1 mM DTT, 10 mM EDTA, 0.2% Triton X-100) and incubated with dT-cellulose at 4ºC for 1 hour. Bound fusions were washed with washing buffer (0.3M NaCl, 20 mM Tris pH 8.0) and eluted by ddH_2O.

b. Reverse transcription and selective step

Fusion after dT purification was concentrated and used for reverse transcription with Superscript II RNase H^- reverse transcriptase (BRL, life Technologies) following standard conditions recommended by the manufacturer. The reaction mixture (50 μl) was directly added into 1 ml phosphate buffer (50 mM, pH 7) and streptavidin-agarose matrix (Pierce). After a 1-hour incubation at 4ºC, the matrix was washed with washing buffer (50 mM phosphate pH 8.0, 100 mM NaCl, 0.1% SDS) 500 μl × 6 times. The matrix then was used for PCR amplification with sd2 and sd26. The PCR product was cloned with TOPO Clone kit (Invitrogen) for sequencing.

Figure 3.6 General scheme to prepare unnatural amino acid charged tRNA. The amine group of amino acid is protected by 4,5-methoxyl-2-nitrobenzyle group first, then its carboxylic group is activated by chloroacetonitrile. The activated amino acid is coupled to dinucleotide pdCpA and ligated to truncated tRNA by T4 RNA ligase.

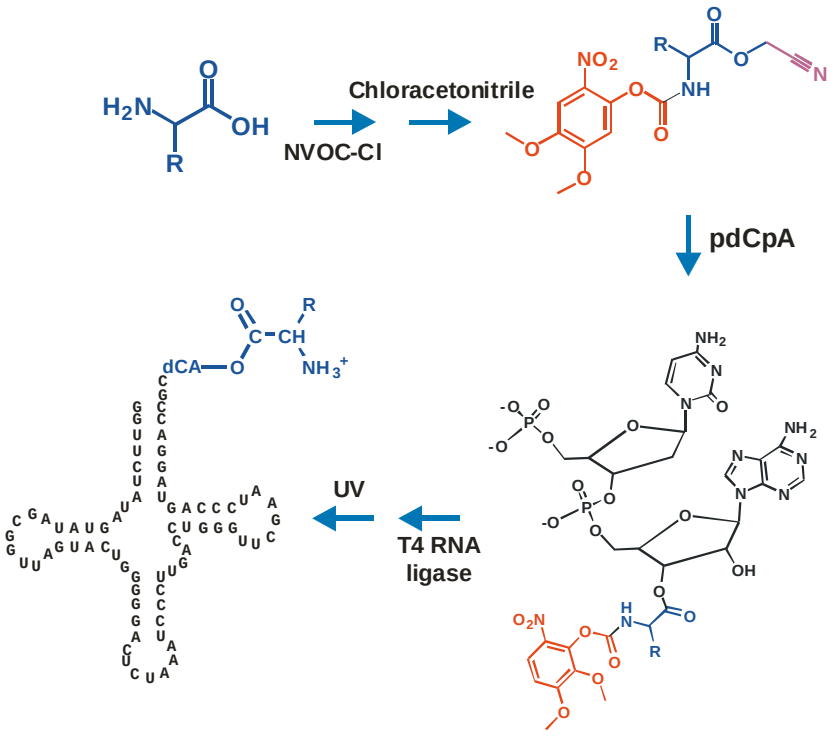
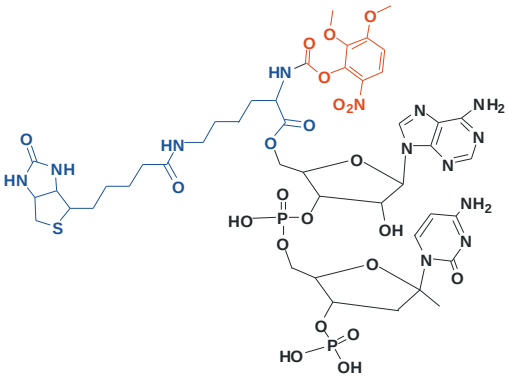
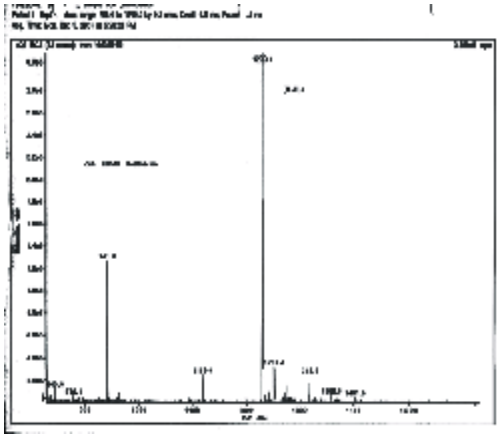


Figure 3.7 Confirmation of biocytin-pdCpA by MALDI-MS.



$C_{45}H_{61}N_{13}O_{22}P_2S$
Exact Mass: 1229.33
Mol. Wt.: 1230.05



Oligo Sequences:

Pep1

5’-ACTATTTACAACCACCATGGGCCGCCAGGAGATCCACTGGGCCAACGA
CCTGTGCAAGCCCTTCTGGGTGTACACCTCC

Pep2

5’-CTATTTACAACCACCATGGGCTAGCTTGACTACAAGGACGAGGACAAGCG
CCAGGAGATCCACTTGGCCAACGACCTGTGCAAGCCCTTCTGGGTGTACACC
TCC

Lib1

5’-ACTATTTACAACCACCATGGGCCGCCAGGAGATCCACNNSGCCAACGACC
TGTGCAAGCCCTTCTGGGTGTACACCTCC
N: 30% A, 30% C, 20% G, 20% T

Sd2

5’-GGATTCTAATACGACTCACTATAGGGACAATTACTATTTACAACCACCATG

Sd4

5’-TTTTTTTTTTTTNGCCGCCGCCGCC
N: 75% T; 9.4%A, 9.4% G; 6.2% C

Sd26

5’-GCCGCCGCCGCCGGAGGTGTACACCCAGAAG

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Chapter 4

Catalytic selection of b-lactamase with mRNA display library

Abstract: A novel catalytic selection approach for improvement of enzymatic function is demonstrated. An immobilized mechanism-based inhibitor, whose inhibition stimulated by the same mechanism of enzyme as to normal substrate, is able to enrich the active mutants of enzyme quickly and efficiently. Biotinylated penicillanic acid sulfone, a β-lactamase mechanism-based inhibitor, is employed to select active mutants of β-lactamase from an mRNA-lactamase fusion library. This strategy can be applied on the functional evolution of other enzymes, such as aldolase.

4.1 Introduction

1. Functional evolution of enzymes

The enzymes present in modern biochemistry catalyze a great variety of chemical reaction precisely and efficiently as a result of billions of years of natural evolution. A clear understanding of their structures, mechanisms and functions is important to the advancement of medicine, synthetic chemistry, material science, as well as many other fields. Despite tremendous efforts, many aspects of enzymes remain a mystery. One example is the minimum size necessary to keep their active site residues in their proper positions for catalysis. Indeed, the smallest enzymes known tend to be ~100 residues, for example, acylphosphatase[1], a 98-residue-long enzyme. It is unclear if short catalytic proteins existed in the early stages of evolution and then were replaced by larger enzymes due to the demands of high efficiency and precision. If the existence of short catalytic proteins that mimic the function of natural enzymes can be demonstrated, it will lend insight into the origins of life. Despite the failure of many de novo efforts to produce such short protein catalysts with biologically interesting properties[2,3], the advancement of combinatorial approaches including enzyme evolution provides an attractive alternative.

Enzyme evolution has been used widely to improve key properties of many enzymes, such as substrate specificity and thermo stability[4,5]. Generally, enzyme evolution starts with the creation of a library of mutated genes. Mutants with enhanced properties are identified from the background of neutral or harmful mutants, and subjected to further cycles to accumulate beneficial mutations. Currently, screening is the primary method to identify positive mutants because it is easy to implement and apparently suitable to any enzymes, as long as the size of libraries is kept manageable.

However, to manipulate even a small size of library is not trivial. Even though the advance of robotic systems in recent years already exceedingly facilitates the screening process, the size of libraries that can be handled by screening is still its major limitation. *In vivo* selection, by contrast, has apparent advantages over screening for searching larger libraries. Nevertheless, the *in vivo* selection suffers from its difficulty to be used in an enzyme that is not critical to the survival of the host organism. In addition, organisms are notoriously adept at evading imposed selective pressure by unexpected mechanisms. In stead, *in vitro* selection can be employed on any molecules that are capable of being copied. The stringency of selection pressure is also controllable *in vitro*. Thus, an *in vitro* catalytic selection strategy based on mRNA display library is reported herein.

2. Catalytic selection with mechanism based inhibitors

Affinity selection has been extensively utilized to recognize the epitopes for antibodies, detect new receptors with natural ligands, and select DNA-binding proteins in either *in vivo* or *in vitro* libraries. A variation of affinity selection, where the transition state analogs of chemical reactions are used as antigens, can generate catalytic antibodies[6]. This technique is based on the fact that the antibodies specific for the transition state analog of chemical reactions should have catalytic power. However, catalytic antibodies are usually much less efficient than natural occurring enzymes. In many cases, antibodies bound to the transition state analog tightly fail to accelerate the chemical reaction. Recently, reactive molecules during immune response have been used as antigens to generate catalytic antibodies[7-9]. This approach has proven to be superior over the conventional catalytic antibody selection. Nevertheless, the immune system has

several limitations: 1) the scaffold for recombination is limited to the immunoglobin family, 2) the selection stringency cannot be controlled, and 3) the optimization of selected antibodies is difficult. Thus, the concept of "reactive immune response" is applied on mRNA-display library to select active enzymes from a library instead.

In this new strategy, the immobilized mechanism-based inhibitor, which is defined as an unreactive compound that inhibits an enzyme upon being activated, is employed as target to search the mRNA display library. A mechanism-based inhibitor, which shares structural similarity with the substrate or product of a specific enzyme, is converted to an intermediate product that is generally very reactive to target enzyme. While in the active site, this active intermediate quickly forms a covalent bond with target enzyme, thus inactivating the enzyme. The elegant inhibitory mechanism of mechanism-based inhibitor differs from that of normal competitive or non-competitive inhibitors in that a chemical reaction via the enzyme's normal catalytic pathway must take place to activate the mechanism-based inhibitor. This mechanism provides a powerful method for catalytic selection. When the entire mRNA display library of a specific enzyme is incubated with the immobilized mechanism-based inhibitor, only mutants maintaining the normal functions are able to form covalent bond with inhibitor and bind to solid support. All other inactive individuals are removed by one simple washing step. After identifying functional mutants from the library, error prone PCR can be used to add diversity to the library prior to the next cycle of selection.

3. A model system – b-lactamase

RTEM-1 β-lactamase, a 30 kD enzyme, has been chosen as a model system because of its unique features, including the simplicity of its structure, extensive understanding of its mechanism, and the availability of its mechanism-based inhibitors.

β-lactamases are a wide family of proteins encoded by either chromosome or plasmid. These enzymes are normally responsible for the bacterial resistance towards β-lactam antibiotics because they can hydrolyze β-lactam ring efficiently. RTEM-1 β-lactamase is a plasmid-encoded enzyme that consists of only 263 amino acid residues in a single polypeptide chain[10]. It has attained a kinetic optimum in that its catalytic activity towards penicillin is diffusion-limited.

a. Mechanism of RTEM-1 b-lactamase

Extensive investigations on the mechanism of RTEM-1 β-lactamase indicate that a serine residue in position 70 plays an essential role in catalytic reaction[11] (Figure 4.1). Catalysis is initiated by nucleophilic attack of Ser70 on the β-lactam ring to form a tetrahedral acyl intermediate. Then, the product is released by the nucleophilic attacking of a water molecule, which is activated by Glu166. The E166A mutant of β-lactamase is hydrolysis-deficient[12,13].

b. Penicillanic acid sulfone (PAS) – a mechanism-based inhibitor of b-lactamase

Penicillanic acid sulfone is a potent mechanism-based inhibitor of β-lactamase[11,14-17] (Figure 4.2). Inhibition starts from nucleophilic attack of Ser70 on the β-lactam ring to form an acyl-enzyme intermediate. However, the pentacyclic ring of penicillanic acid sulfone is labile because the strong electron-withdrawing sulfone group

can stabilized the cleaved product. Thus, the acyl-enzyme intermediate can follow three possible pathways[18]. The first is normal deacylation, which regenerates the enzyme. The

Figure 4.1 Two-step mechanism of RTEM-1 β-lactamase.

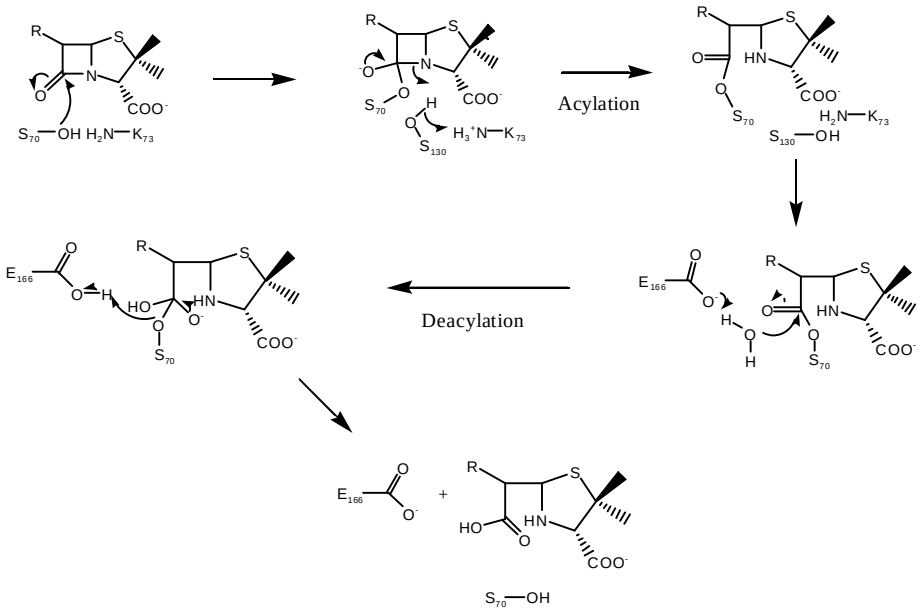
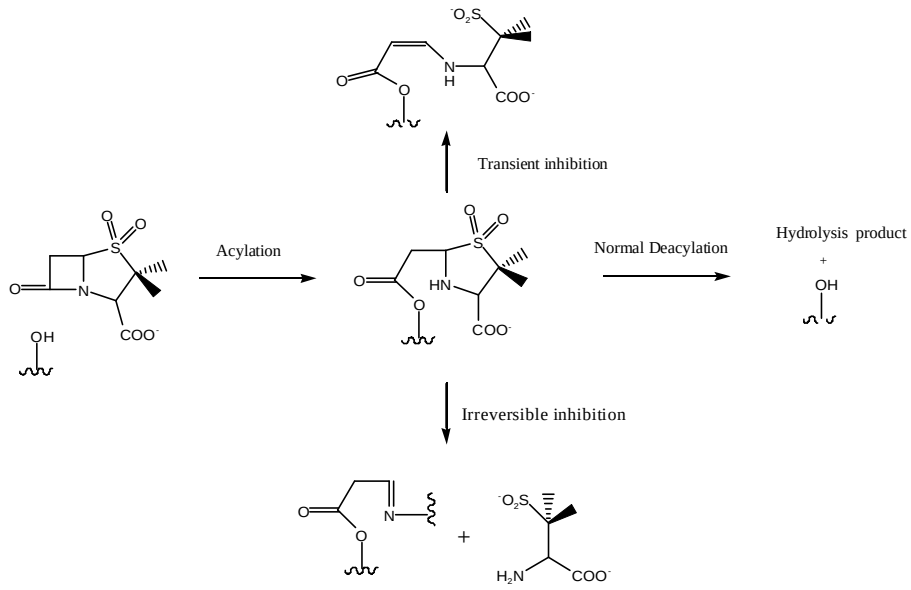


Figure 4.2 Inhibitory mechanism of penam sulfone.



second pathway is transient inhibition where the acyl-enzyme intermediate is tautomerized to another much more stable enamine intermediate. The enamine intermediate can be slowly released from the active site to accomplish the turnover of β-lactamase. As the last possibility, the inhibitor forms an intermolecular crosslink with the ε-amine of a lysine proximal to the active site, presumably either Lys73 or Lys234[19], accompanied by the concomitant release of penicillamine sulfuric acid. Thus the activity of β-lactamase is totally lost due to this irreversible inhibition. It was reported that partition of acyl-enzyme intermediate in these three pathways depends on the nature of 6-substituent on the β-lactam, as well as the pH of reaction condition. Generally, in lower pH, the irreversible inhibition is more efficient[20]. Electron-withdraw substituents on the 6-position of β-lactam also facilitate the formation of irreversible crosslink product.

4. Methodology

The catalytic selection involves three steps: 1) the generation of fusion library, 2) the preparation of immobilized inhibitor, and 3) the selection scheme.

a. Generation of mRNA-protein fusion library

The diversity of the β-lactamase fusion library is introduced by error-prone PCR. The mutation rate in each base of the DNA template is controlled as 1.0%[21]. Thus, the resulting protein library has a high percentage of multiple mutations. The probability P of having k mutations in a sequence of length n can be calculated using equation below (Figure 4.3 A).

$P(k,n,e) = (n!/[(n-k)!k!])e^{k}(1-e)^{n-k}$, where ε is the error rate per position.

In a library containing 10^{13} different sequences, the fraction of coverage of N-sites mutants, defined as the number of N-site mutants in library divided by the total possible recombinants of N-site mutants, can also be calculated (Figure 4.3 B). The high mutation rate can guarantee the exploration of multiple mutations with compensatory, additive or co-operative effects[22]. Evolution under low mutation rate, however, can only survey additive mutants in the library.

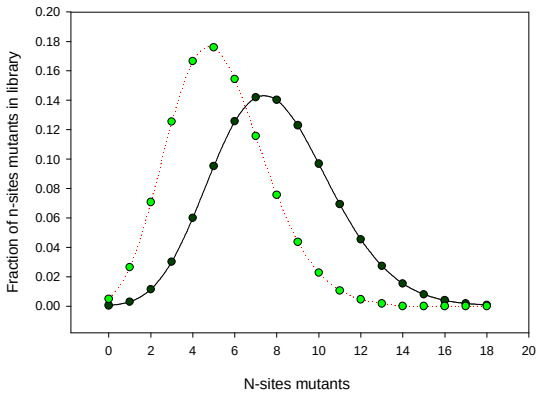
b. Preparation of immobilized inhibitor

Biotinylated penicillanic sulfone is used as the immobilized mechanism-based inhibitor of β-lactamase (Figure 4.4). The biotin moiety allows the inhibitor to be immobilized on the streptavidin agarose gel under harsh conditions. The long polyether chain provides space to avoid potential interference of the solid support on the catalytic reactions. In addition, the existence of a disulfide bond makes it feasible to liberate the immobilized enzymes from the streptavidin agarose under mild reductive conditions.

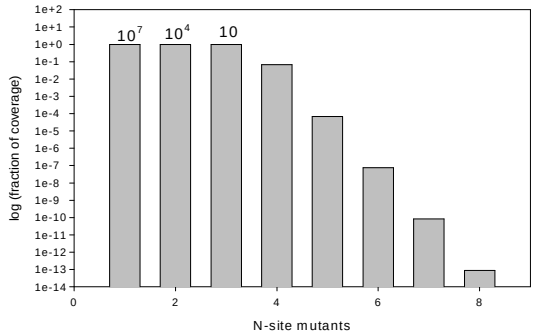
c. Scheme for selection of b-lactamase turnover

Following the generation of β-lactamase fusion library, the entire library is incubated with the biotinylated penicillanic sulfone in the presence of ampicillin. Both active mutants that can regenerate themselves and mutants with the properties of penicillin binding proteins have the capability to form covalent link with inhibitor. Thus these two types of mutants can not be distinguished if only biotinylated penicillanic sulfone is used. The presence of excess ampicillin, the substrate of β-lactamase, can prohibit the mutants similar to penicillin binding proteins from binding with inhibitors[23]. Only mutants that are able to turnover can be immobilized and subjected for further cycles of selection.

Figure 4.3 [A]. Distribution of N-site mutants in β-lactamase library. The size of DNA template is 789 bp and mutation rate is 1.0% base. The black points represent the number of mutants in DNA template and the green points represent the number of mutants in respective protein. [B]. Log(fraction of coverage) to N-site mutants. The fraction of coverage is defined as the number of N-site mutants in a library containing 10^{13} different sequences divided by the total possible recombinants of N-sites mutants when the mutation rate is 1% for β-lactamase DNA template. For 1- 2- 3-site mutants, the number of copies that have the same sequence is show on the top of respective columns.

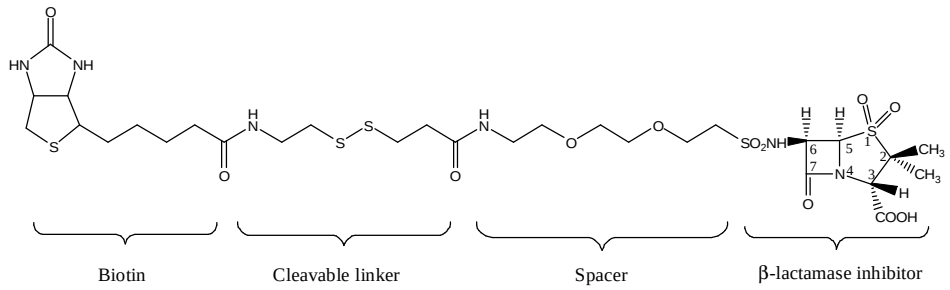


[A]

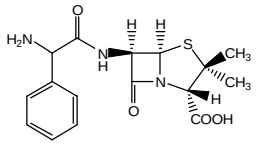


[B]

Figure 4.4 Structure of biotinylated penicillanic sulfone, a mechanism based inhibitor of β-lactamase. As a comparison, the structure of ampicillin, a substrate of β-lactamase, is also drawn.



biotinylated penicillanic acid sulfone



Ampicillin

4.2 Results and discussion

Several technical issues have impeded the accomplishment of this catalytic selection. For example, although the protocol for making small mRNA-peptide fusions is quite mature, the preparation of mRNA-lactamase fusion proved to be a new challenge due to relatively large size of β-lactamase (263 amino acid residues) and its encoding mRNA (814 oligonucleotides). The nature of large β-lactamase imposes three problems: 1) the secondary structure prevents the efficient ligation of mRNA with the short puromycin oligomer, 2) ligated mRNA is inseparable from unligated mRNA in polyacryamide gel, and 3) the fusion formation from ligated mRNA is inefficient. To enhance the ligation, the oligomer mixture in water was pre-heated to disrupt secondary structures. The sample was then cooled down in ice to allow the annealing between complementary sequences. The second problem can be solved by a two-step separation, gel purification followed by dT-cellulose affinity chromatography. Nevertheless, the optimization of conditions was unable to improve the fusion formation.

The activity assay for mRNA-linked β-lactamase was also problematic due to the limited amount of fusion. The standard nitrocefin assay was not sensitive enough to test such low amount of enzyme. Thus, whether the mRNA-linked β-lactamase is functional or not is still a mystery.

The purpose of this project is to address the catalytic selection with mRNA display library. The mRNA display β-lactamase is by far the largest mRNA protein fusion made in our lab. The lessons we have learnt include 1) the mRNA display technique is inefficient for large protein fusion, and 2) the appending mRNA template

may impair the activity of its encoding enzyme. Nevertheless, a recent similar research on ribosome display library indicates the catalytic selection with immobilized mechanism-based inhibitors is a powerful approach to identify functional mutants of enzymes. Future work of this unfinished project will focus on the identification of the "smallest" catalytic polypeptides from a fusion library containing ~100 amino acid residues.

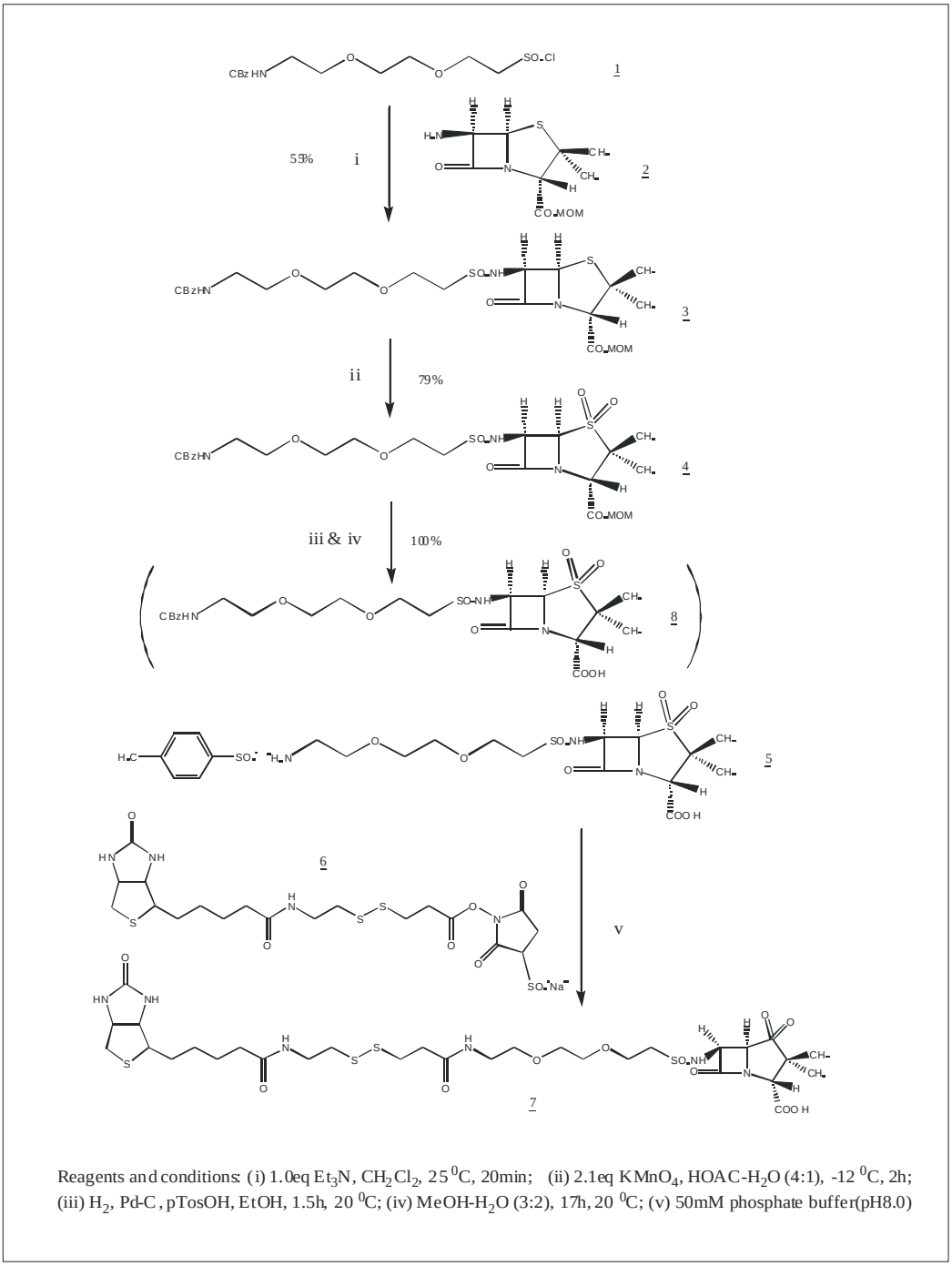
The existence of catalytic RNA has already shed a light on the early evolution of life. However, the evolution of enzymes remains enigmatic. Was the takeover of catalysis from RNA by proteins originally performed by peptides or were "modern"-sized proteins recruited to play this role? We hope to answer these questions by examining the abundance of short protein catalysts in future.

4.3 Materials and methods

1. Synthesis of biotinylated penicillanic acid sulfone

The synthesis of biotinylated penicillanic acid sulfone follows the procedure described previously (Figure 4.5). MOM (methoxymethyl) protected 6-aminopenicillanic acid 2 is used as the starting material to give penam derivative 3. Subsequent oxidation of 3 with $KMnO_4$ results in the protected penicillanic acid sulfone 4. MOM deprotection by mild hydrolysis quantitatively yields the free acid 8. Instead, hydrogenation of protected penicillanic acid sulfone 4, followed by deprotection of MOM, can give the total deprotected penam sulfone 5. This sulfone reacts with biotin-SS-NHS 6 to give the final product biotinylated penicillanic acid sulfone 7. Since the p-toluenesulfonate counter anion in the totally deprotected penam sulfone 5 has a high UV absorbance at

Figure 4.5 Synthesis pathway of biotinylated penam sulfone.



Reagents and conditions: (i) 1.0eq Et_3N, CH_2Cl_2, 25 ^{0}C, 20min; (ii) 2.1eq $KMnO_4$, HOAC-H_2O (4:1), -12 ^{0}C, 2h; (iii) H_2, Pd-C, pTosOH, EtOH, 1.5h, 20 ^{0}C; (iv) MeOH-H_2O (3:2), 17h, 20 ^{0}C; (v) 50mM phosphate buffer(pH8.0)

235nm that interferes with the inhibitory measurement, the partial deprotected penam sulfone 8 was used to examine its inhibitory competence. More than 70% activity of β-lactamase is lost after 3 hours incubation with inhibitor at pH 4.5 (Figure 4.6 A). However, the inhibitor is unable to inactive β-lactamase at pH 7.0 because the normal deacylation pathway is favored at high pH (Figure 4.6 B).

2. Preparation of mRNA-lactamase fusion

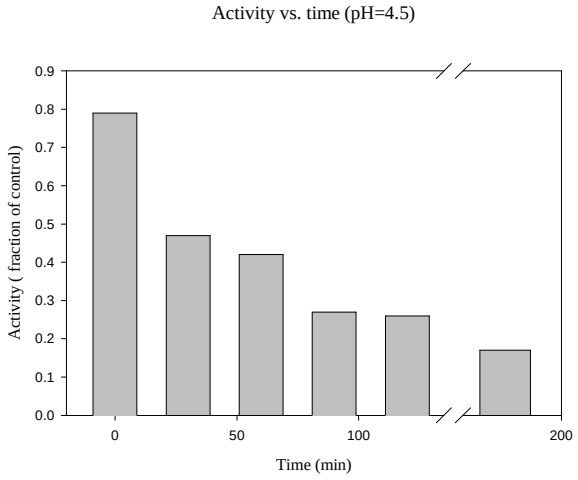
a. Preparation of b-lactamase DNA template

RTEM-1 β-lactamase DNA template is amplified from a plasmid pET22b (Novagene) by polymerase chain reaction (PCR), using two synthetic oligomers (P063a01 and P028a01) as primers. The composition of P063a01 includes a T7 promoter sequence, a sequence adopted from the 5' untranslated region (5'-UTR) of tobacco mosaic virus, and the first 18 nucleotides of β-lactamase open reading frame. The P028a01 is complementary to the last 28 nucleotides of β-lactamase. The PCR reaction buffer contains 10mM Tris-HCl (pH9.0 at 25°C), 50mM KCl, 0.1% Triton X-100, 1.5mM Mg^{2+}, 200 μM each dNTP, 1 μM each primer, 20ng pET22b, and 2.5U Taq polymerase in per 100 μl solution. The PCR reaction is conducted in a thermal cycler (MJ research) with the program: 3 mins at 94°C followed by 20 cycles of 1 min at 94°C, 1 min at 55°C, 2 mins at 72°C, and ended with 5 mins at 72°C.

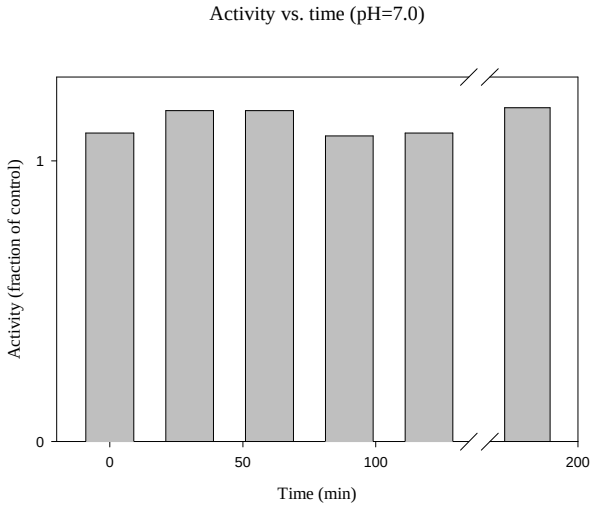
b. In vitro transcription of b-lactamase DNA template

The transcription reaction is accomplished in the buffer including 80mM HEPES-KOH (pH7.5), 40mM DTT, 25mM Mg^{2+}, 4mM each NTP, 0.2nM DNA template, and 10U/ml T7 RNA polymerase. The reaction mixture is incubated at 37°C for 3 hours,

Figure 4.6 [A]. Inhibition of β-lactamase by penicillanic acid sulfone at pH4.5. The remaining activity of β-lactamase is taken as the fraction of the activity of β-lactamase with inhibitor over that of β-lactamase without inhibitor. [B]. Inhibition of β-lactamase by penicillanic acid sulfone at pH7.0.



[A]



[B]

then 1/10 volume of EDTA (500mM, pH8.0) is added to dissolve magnesium pyrophosphate precipitate. The supernatant is extracted with phenol/chloroform (50:50) mixture and subjected to ethanol precipitation. The mixture is incubated at –70°C for 30 min or at –20°C for overnight. After spinning at 15000 RPM for 10 min at 4°C, the pellet is washed with 70% ethanol twice and dried in air, then dissolve in DEPC-treated water. The RNA template is transferred to a size exclusion column (NAP-25, Pharmacia Biotech) to remove nucleotides.

c. Ligation of b-lactamase mRNA with puromycin oligonucleotides

A short oligomer 30-P with a 3'-puromycin is synthesized from puromycin-CPG (Glen Research). The β-lactamase mRNA ligates with 30-P with T4 DNA ligase in the presence of a splint template (P030a02). The ligation reaction consists of 50mM Tris-HCl (pH 7.8 at 25°C), 10mM $MgCl_2$, 10mM DTT, 1mM ATP, 25 μg/ml BSA, 15 μM 30-P, 15 μM mRNA, 30 μM P030a02, and T4 DNA ligase at a concentration of 2U per picomole of mRNA. The mixture of oligomers is heated at 90°C for 30 secs first and cooled down for 15 min at ice. After adding T4 DNA ligase and ligation buffer, the reaction is performed at room temperature for 1 hour.

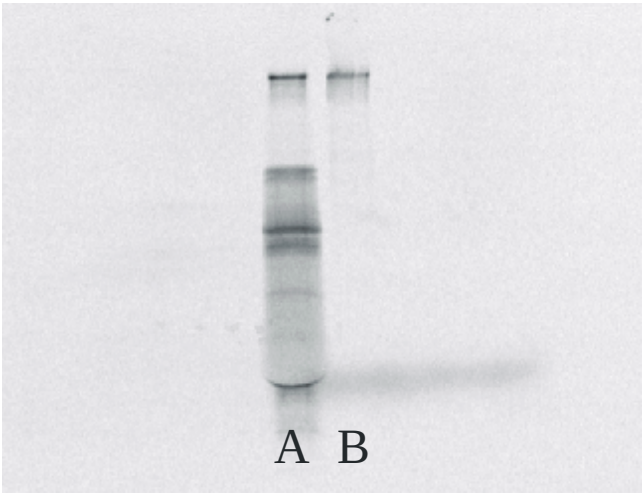
Two steps are required to purify ligated mRNA from unligated mRNA because they are indistinguishable even in 5% urea-PAGE (polyacrylamide gel electrophoresis). First, both ligated mRNA and unligated mRNA are separated from unreacted 30-P and P030a02 by PAGE. Then, ligated mRNA is purified with dT-cellulose (New England Biolab). The sample is shaken with dT-cellulose for 1 hour at 4 °C in binding buffer (1M NaCl; 20mM Tris-HCl, pH 8.0; 10mM EDTA; 1 mM DTT; 0.2% Triton X-100). After washing with 10 volume of binding buffer, following with 10 volume of washing buffer

(0.3 M NaCl; 20mM Tris-HCl, pH 8.0; 1 mM DTT), pure mRNA is eluted from dT-cellulose with water. A quick spin of elution in 0.2 μm filter unit easily removes the fine powder of dT-cellulose resin.

d. In vitro translation of b-lactamase

Translation reaction is performed in reticulocyte lysate (Novagene) by following conditions recommended by manufacturer. Upon the completion of translation, Mg^{2+} in mixture is adjusted to 50mM and K^{+} to 0.6M. The mixture is then kept at –20°C for 24 hours for dT-cellulose purification and SDS-PAGE analysis (Figure 4.7).

Figure 4.7 Preparation of mRNA-lactamase fusion. Lane A: fusion in rabbit reticulocyte lysate; Lane B: fusion after dT-oligo purification.



3. Inhibitory measurement of partial deprotected penicillanic acid sulfone 8

3 mg partial deprotected penicillanic acid sulfone 8 was dissolved in 500 μl mixture of 95% water and 5% DMSO. 25 μl inhibitor solution (10mM in 5% DMSO) was added to 75 μl reaction solution (NaOAC, 100mM, pH 4.5) containing β-lactamase at room temperature. 2 μl portions were taken out at an interval of 30 minutes and diluted into 100 μl of ampicillin solution (1mM; 50mM phosphate, pH 7.0). The enzyme activity was recorded for 5 minutes as the UV absorbance at 235nm decreases. As a control, another β-lactamase solution without the inhibitor was tested the same way. The remaining enzyme activity in the presence of inhibitor was calculated as the fraction of control enzyme's activity. All kinetic studies are conducted at 25°C.

Oligo sequences:

P063a01

5’-TAATACGACTCACTATAGGGACAATTACTATTTACAACTACCATGCACCC
AGAAACGCTGGTG

P028a01

5’-CCGCTCGAGCCAATGCTTAATCAGTGAG

P030a02

5’-TTTTTTTTTTTTTTNCCAATGCTTAATCAG

N: 50% T, 16.7% A, 16.7% G, 16.7% C

F30P

5’-d$A_{21}[C_9]_3$dACdCP;
C_9=triethylene glycol phosphate
P = puromycin

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