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.

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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.

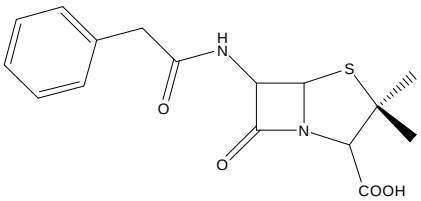
The mRNA display approach allows libraries containing more than 10^{13} independent peptides or proteins to be constructed entirely *in vitro*, providing the highest sequence diversity currently available with any method[13,14]. Additionally, because all steps in the mRNA display process are conducted *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).

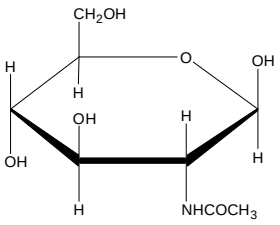
2. Penicillin antibiotics and their mechanism

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.

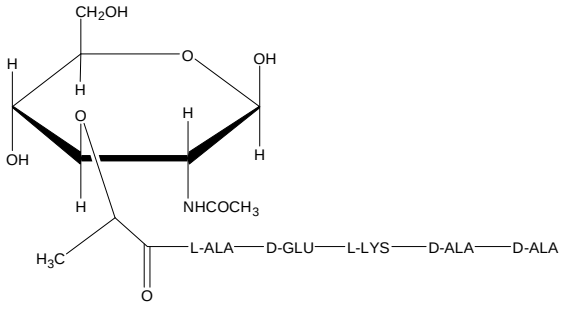


Structure of a typical penicillin drug benzyl-penicillin

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)



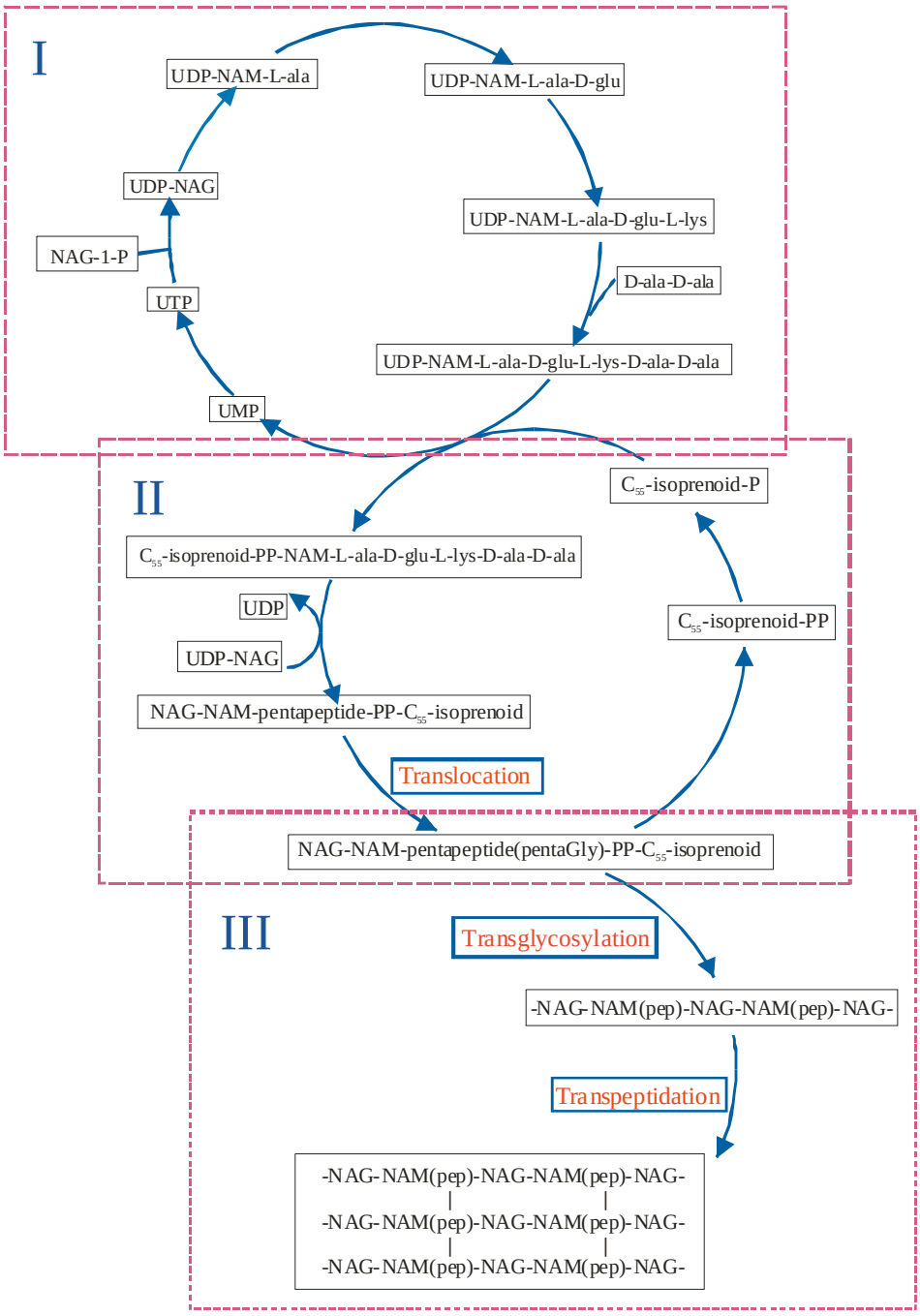
N-acetylmuramic acid-peptide (NAM)

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).

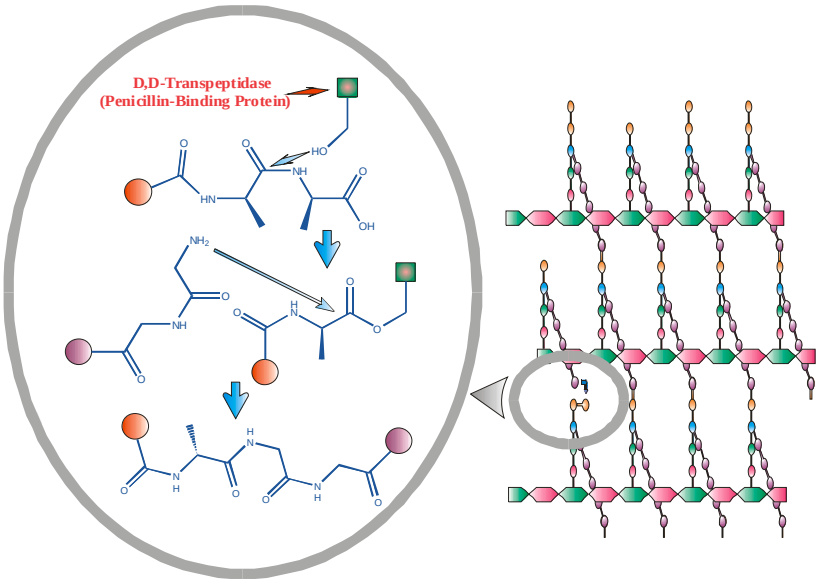


Figure 2.3 The conformation similarity between penicillin, cephalosporin and D-alanyl-D-alanine.

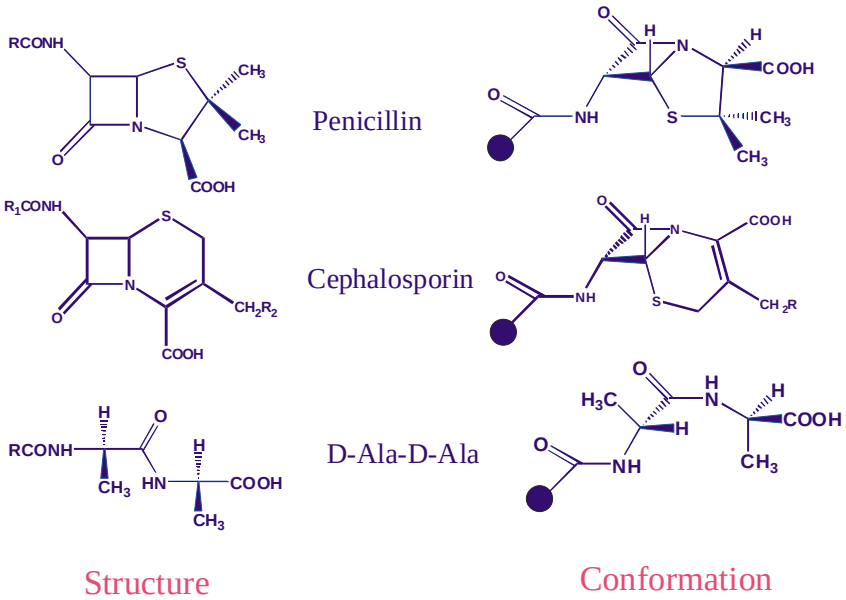
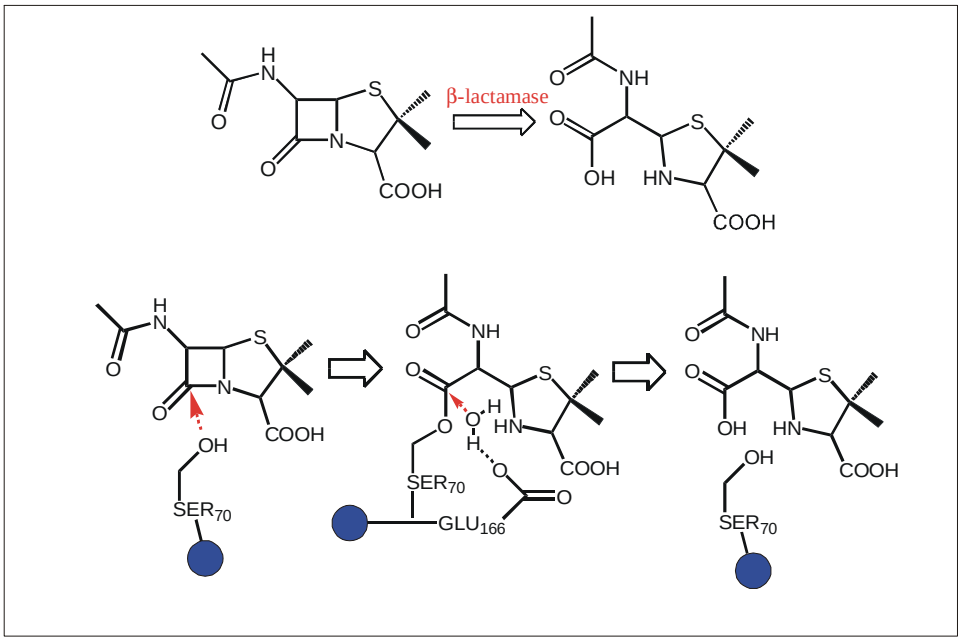


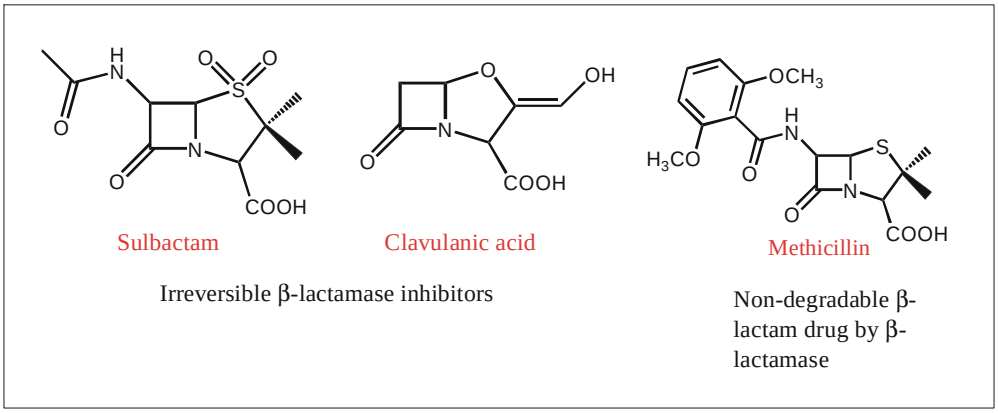
Figure 2.4 The mechanism of degradation of β-lactam compounds by β-lactamase.



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.

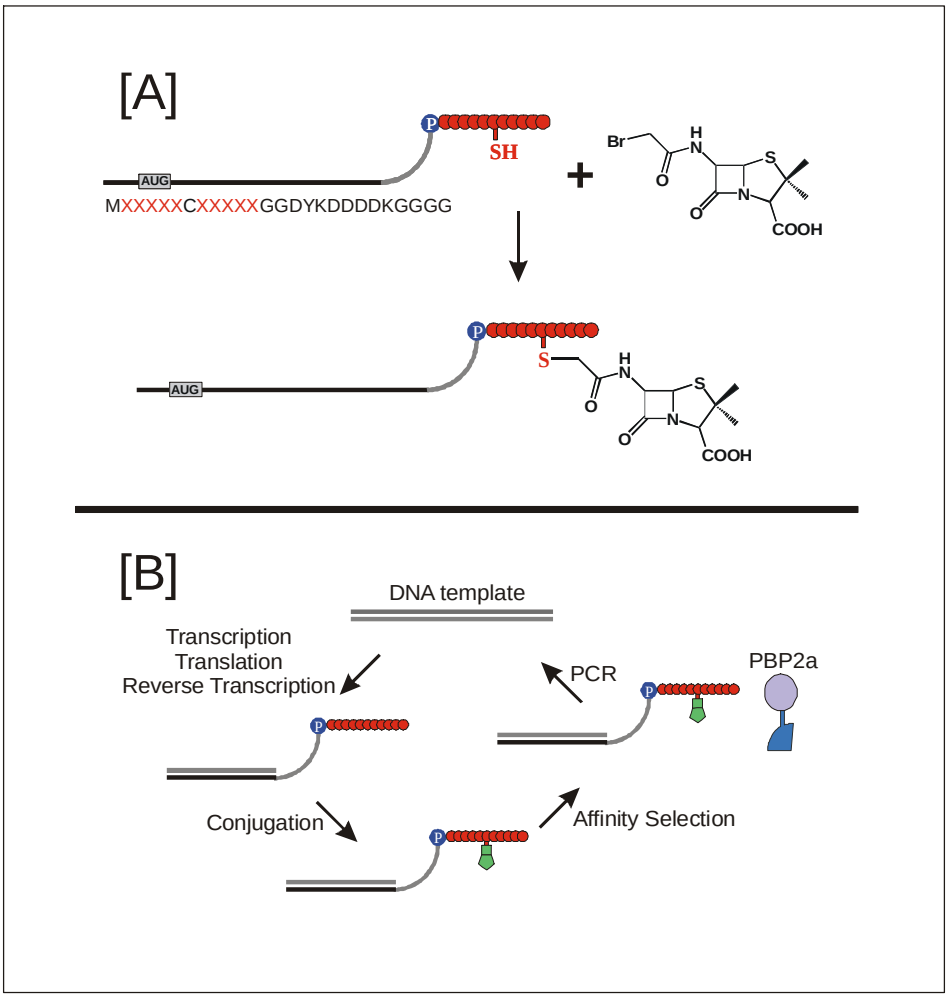
Another type of β-lactam compound, cephalosporin, differs from penicillins with a 6-member ring fusing with the core β-lactam (Figure 2.3). Recently, it was indicated that substituted cephalosporin ring has additional *van der Waals* contacts with the active site of PBP2a, providing higher affinity than 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.



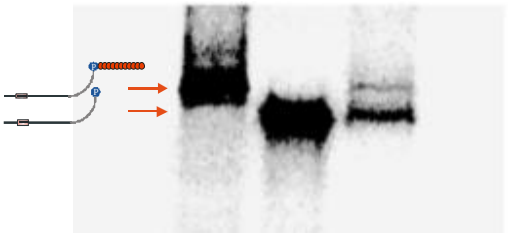
2.2 Results and discussion

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).

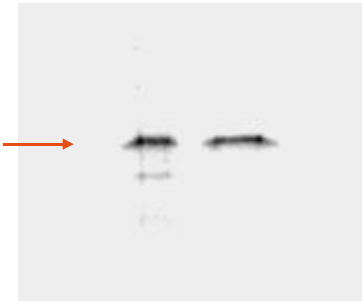
After coupling the library with sodium 6-bromoacetyl penicillanate, we needed to address two issues: (1) the fraction of the library bearing the drug and (2) whether any other functional groups on the peptide, RNA, or DNA were reactive with bromoacetyl moiety. The drug-modified mRNA display library is 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

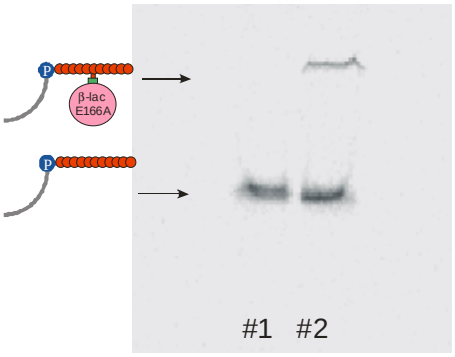
[A]



fusion fusion + thiopropyl gel

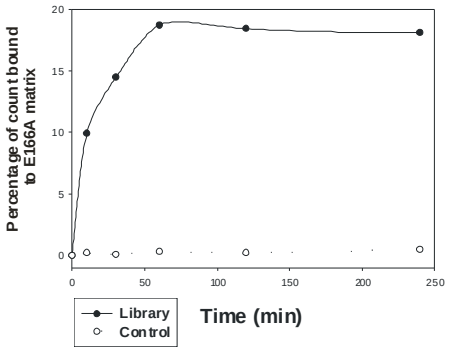
[B]

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.

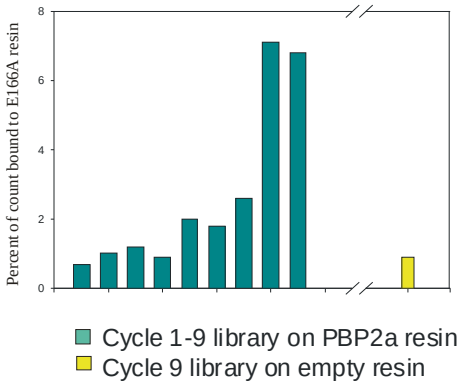
2. Selection against immobilized PBP2a

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.

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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--..........
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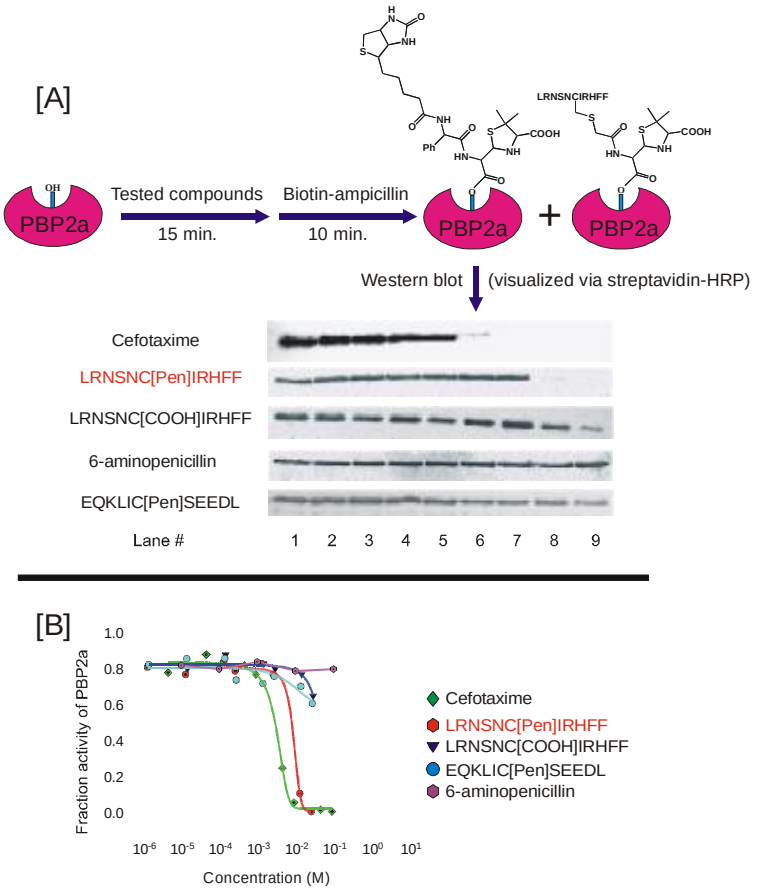
[A] **[B]**

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.

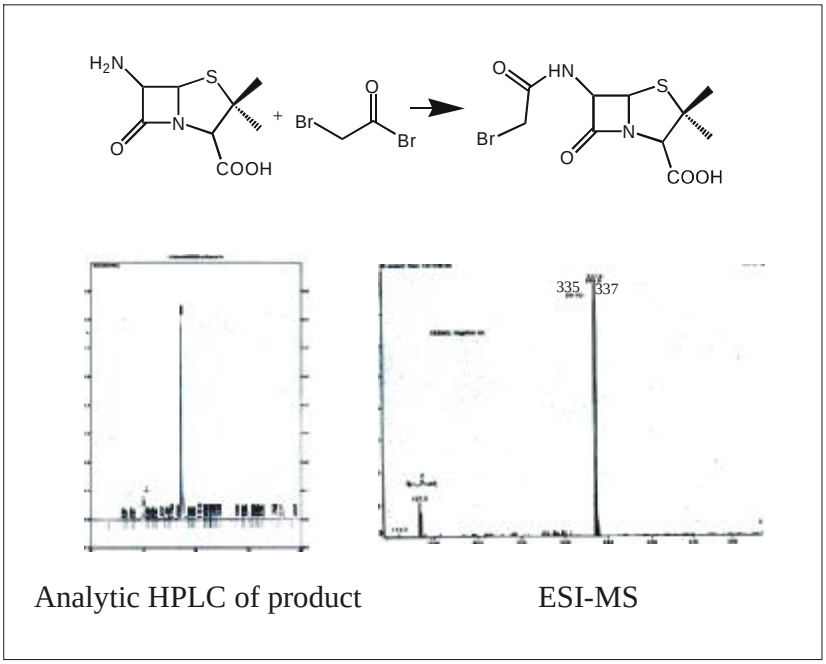


Figure 2.12 Synthesis of fluorescein-ampicillin.

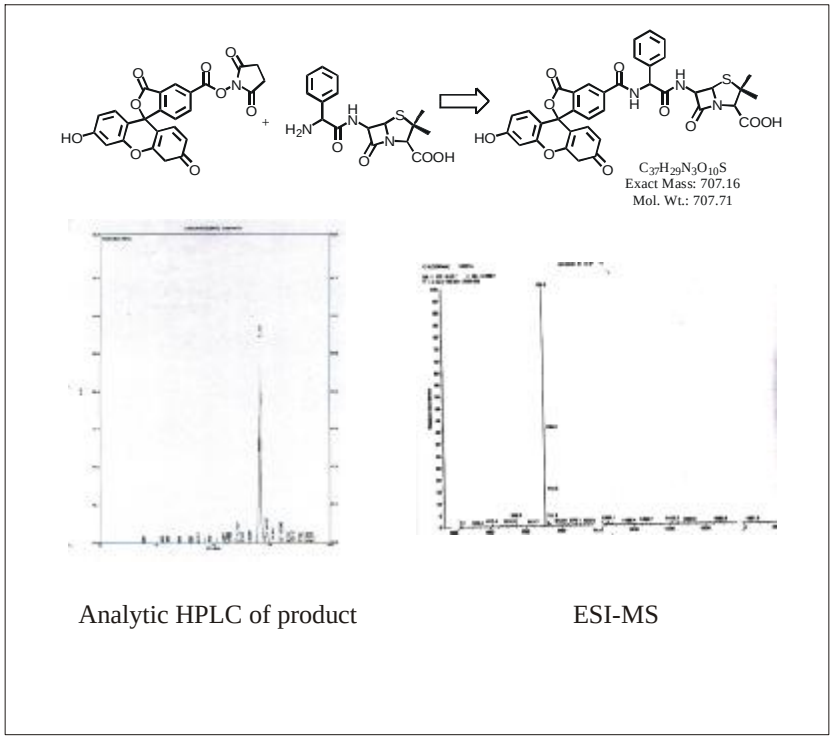
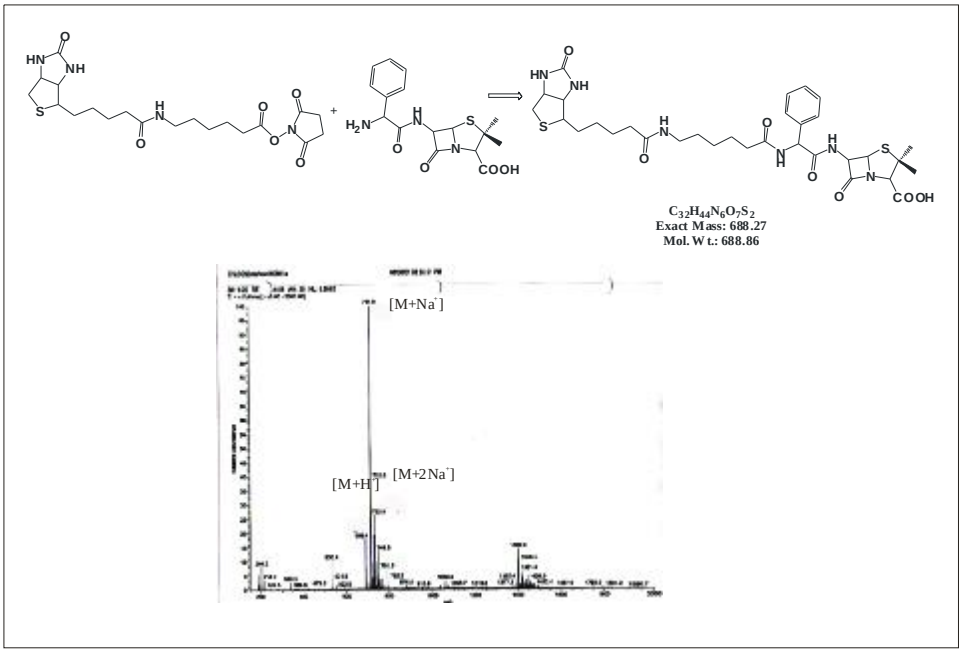


Figure 2.13 Synthesis of biotin-LC-ampicillin. The product is confirmed by mass spectrum.



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

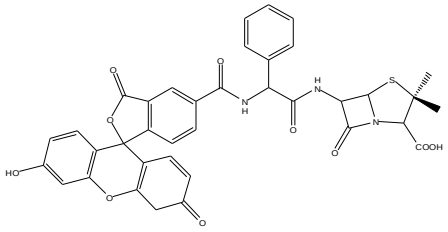
Plasmid bearing PBP2a gene was a gift from Professor Hiramatsu (Juntendo University, Tokyo, Japan). PBP2a gene was amplified by PCR with sd14 and sd16 as primers. The resulting DNA template was cut by restriction enzymes NcoI and SapI (New England Biolab) and ligated into PTXB3 plasmid (New England Biolab) 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

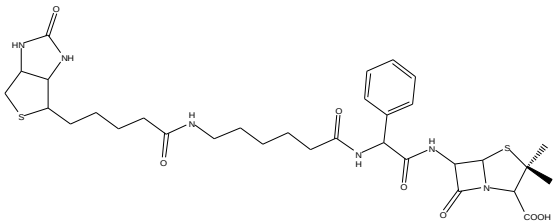
The purified PBP2a was tested for activity by reacting with fluorescein-ampicillin conjugate. After 30 minutes incubation, the mixture was directly loaded onto SDS polyacryamide gel. The activity of PBP2a was confirmed by the green fluorescence appearing at the position where PBP2a was (80 kD) under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