

**IMPROVING THE ENZYMATIC SYNTHESIS OF SEMI-SYNTHETIC  
BETA-LACTAM ANTIBIOTICS VIA REACTION ENGINEERING  
AND DATA-DRIVEN PROTEIN ENGINEERING**

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*For Grandpa*

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## NOMENCLATURE

1P1S	one-pot, one-step
1P2S	one-pot, two-step
6-APA	6-aminopenicillanic acid
6-APA:( <i>R</i> )-PGA	ratio of 6-aminopenicillanic acid to ( <i>R</i> )-phenylglycine amide
6-APA:( <i>R</i> )-PGME	ratio of 6-aminopenicillanic acid to ( <i>R</i> )-phenylglycine methyl ester
7-ACA	7-aminocephalosporanic acid
7-ADCA	7-aminodeacetoxycephalosporanic acid
ACS GCI	American Chemical Society Green Chemistry Institute
ACS GCIPR	American Chemical Society Green Chemistry Institute Pharmaceutical Roundtable
AEH	$\alpha$ -amino ester hydrolase
AMP	ampicillin
C $_{\alpha}$	alpha carbon
HPLC	high performance liquid chromatography
iPGA	immobilized penicillin G acylase
NIPAB	2-nitro-5-[(phenylacetyl)amino]-benzoic acid
Ntn	N-terminal nucleophile
PAA	phenylacetic acid
PENG	penicillin G
PGA	penicillin G acylase
PMSF	phenylmethylsulfonyl fluoride
( <i>R</i> )-AMP	( <i>R</i> )-ampicillin
( <i>R</i> )-PG	( <i>R</i> )-phenylglycine methyl ester

(R)-PGA	( <i>R</i> )-phenylglycine amide
(R)-PGHEE	( <i>R</i> )-phenylglycine hydroxyethyl ester
(R)-PGME	( <i>R</i> )-phenylglycine methyl ester hydrochloride
<i>rac</i> -PGME	racemic phenyl glycine methyl ester
(S)-AMP	(S)-ampicillin
(S)-PG	( <i>S</i> )-phenylglycine
(S)-PGME	( <i>S</i> )-phenylglycine methyl ester
S/H-ratio	synthesis to hydrolysis ratio
UAmp	one $\mu\text{mol}$ of ampicillin hydrolyzed per minute
UPenG	one $\mu\text{mol}$ of penicillin G hydrolyzed per minute

## SUMMARY

Semi-synthetic  $\beta$ -lactam antibiotics are the most prescribed class of antibiotics in the world. Chemical coupling of a  $\beta$ -lactam moiety with an acyl side chain has dominated the industrial production of semi-synthetic  $\beta$ -lactam antibiotics since their discovery in the early 1960s. Enzymatic coupling of a  $\beta$ -lactam moiety with an acyl side chain can be accomplished in a process that is much more environmentally benign but also results in a much lower yield. The lower yield in the enzymatic synthesis can be attributed to the fact that the enzymes that catalyze the reaction, penicillin G acylase (PGA) or  $\alpha$ -amino ester hydrolase (AEH), have the ability to catalyze the undesired primary hydrolysis of the side chain acyl donor and the secondary hydrolysis of the antibiotic, in addition to the desired synthesis reaction. The goal of the research presented in this dissertation is to improve the enzymatic synthesis of  $\beta$ -lactam antibiotics via reaction engineering, medium engineering and data-driven protein engineering.

Reaction engineering was employed to demonstrate that the hydrolysis of penicillin G to produce the  $\beta$ -lactam nucleus 6-aminopenicillanic acid (6-APA), and the synthesis of ampicillin from 6-APA and (*R*)-phenylglycine methyl ester ((*R*)-PGME), can be combined in a cascade conversion. In this work, PGA was utilized to catalyze the hydrolysis step, and PGA and AEH were both studied to catalyze the synthesis step. Two different reaction configurations and various relative enzyme loadings were studied. In all reaction configurations the two-enzyme system that utilized PGA and AEH outperformed the one-enzyme system that utilized only PGA. The one-pot, two step system, in which the PGA catalyzed hydrolysis of penicillin G was allowed to proceed prior to

addition of the acyl donor and AEH catalyzed synthesis, achieved 47% yield and secondary hydrolysis was minimized by optimizing relative enzyme loadings. The one-pot, one-step system, a batch process in which all substrates and enzymes are present at the beginning of the reaction, achieved 39% yield, but could be advantageous due to its operational ease and faster cycle times. Both configurations present a promising alternative to the current two-pot set-up which requires intermittent isolation of the intermediate, 6-aminopenicillanic acid (6-APA).

Medium engineering is primarily of interest in  $\beta$ -lactam antibiotic synthesis as a means to suppress the undesired primary and secondary hydrolysis reactions. The synthesis of ampicillin from 6-APA and (R)-PGME in the presence of ethylene glycol was chosen for study after a review of the literature. It was found that the yield enhancement observed in syntheses in 30% (v/v) ethylene glycol was dependent on the amount of (R)-PGME used for the reaction. Furthermore, it was discovered that the transesterification product of (R)-PGME and ethylene glycol, (R)-phenylglycine hydroxyethyl ester ((R)-PGHEE), is transiently formed during the synthesis reactions. This never reported side reaction has the ability to positively affect yield by re-directing a portion of the consumption of (R)-PGME to an intermediate that could be used to synthesize ampicillin, rather than to an unusable hydrolysis product. (R)-PGHEE was synthesized and its ability to act as an acyl side chain donor for ampicillin synthesis was confirmed. Also, ampicillin synthesis was performed with (R)-phenylglycine amide, an acyl side chain donor that does not undergo transesterification by ethylene glycol, and the ampicillin yield enhancement in 30% (v/v) ethylene glycol was found to be minimal. Based on these results, there is evidence that transesterification by a co-solvent can



positively affect yield by participating in an in-situ mixed donor process, where both (R)-PGME and (R)-PGHEE contribute to the synthesis of ampicillin.

Protein engineering was utilized to alter the selectivity of wild-type PGA with respect to the alpha carbon of its substrates. This work represents the first time that the selectivity of PGA has been studied to synthesize a diastereomerically pure  $\beta$ -lactam antibiotic from racemic substrates. Using existing crystal structures of PGA, residues were targeted for site-saturation based on their proximity to the alpha carbon of the inhibitor penicillin G sulfoxide. Four residues were identified that had altered selectivity toward the desired product, (R)-ampicillin. The most selective variant,  $\beta$ Phe24Ala, improved the wild-type diastereomeric excess (d.e.) value from 37% to a d.e. value of 98%. Furthermore, our (R)-selective variants improved the yield from pure (R)-PGME up to 2-fold and significantly decreased the amount of secondary hydrolysis present in the reactions. Protein engineering techniques should be pursued to further improve the selectivity of these PGA variants to a d.e. value greater than 99.8% in order to eliminate the need to prepare enantiomerically pure substrates for semi-synthetic  $\beta$ -lactam antibiotics.

Overall, we have expanded the applicability of PGA and AEH for the synthesis of semi-synthetic  $\beta$ -lactam antibiotics. We have shown the two enzymes can be combined in a novel one-pot cascade, which has the potential to eliminate an isolation step in the current manufacturing process. Furthermore, we have shown that the previously reported ex-situ mixed donor synthesis of ampicillin for PGA can also occur in-situ in the presence of a suitable side chain acyl donor and co-solvent. Finally, we have made

significant progress towards obtaining a selective PGA that is capable of synthesizing diastereomerically pure semi-synthetic  $\beta$ -lactam antibiotics from racemic substrates.

# CHAPTER 1

## INTRODUCTION

In 2005, the American Chemical Society Green Chemistry Institute (ACS GCI) and representatives from seven leading global pharmaceutical companies formed the ACS GCI Pharmaceutical Roundtable (ACS GCIPR) and defined their mission as catalyzing “the implementation of green chemistry and green engineering in the global pharmaceutical industry,” acknowledging the fact that the “the pursuit of green chemistry and green engineering is imperative for sustainable business and environment.” The members of the ACS GCIPR voted amide formation avoiding poor atom economy reagents as the highest priority for reactions that companies use now but would strongly prefer better reagents [1]. Included in this group of reactions is the synthesis of semi-synthetic  $\beta$ -lactam antibiotics.

### 1.1 $\beta$ -Lactam Antibiotics

$\beta$ -lactam antibiotics have been in clinical use for more than 60 years and are currently the most widely used group of antibiotics utilized to treat bacterial infections. The common moiety of all  $\beta$ -lactam antibiotics is a 2-azetidinone ring, more commonly referred to as the  $\beta$ -lactam ring, which is responsible for their bactericidal capabilities [2]. There are four sub-families of the  $\beta$ -lactam antibiotic family that are currently used in clinical practice: the penicillins, cephalosporins, carbapenems, and monobactams, which vary in their ring structure around the  $\beta$ -lactam ring to form unique  $\beta$ -lactam nuclei as shown in Figure 1.1. The  $\beta$ -lactam nuclei are bonded to different acyl side chains to form

different semi-synthetic  $\beta$ -lactam antibiotics. Acyl side chain variations affect microbial activity and chemical stability and thus lead to expanded spectra of bactericidal activity and increased bioavailability [3]. In Appendix A, the structure, classification, preparations, and manufacturers of all of the United States Food and Drug Administration (FDA) approved  $\beta$ -lactam antibiotics are listed.

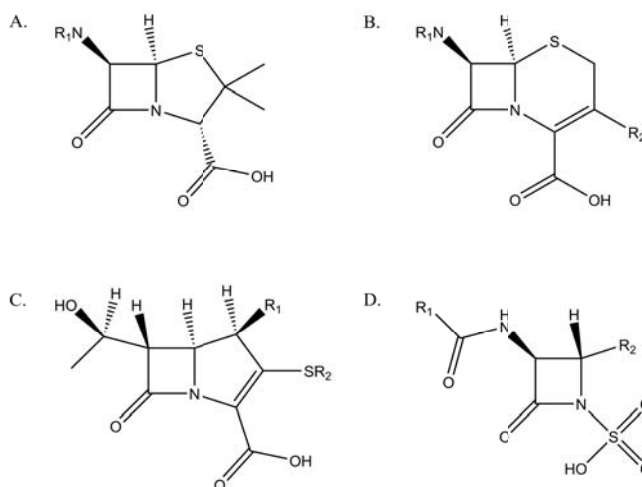


Figure 1.1: The  $\beta$ -lactam nuclei corresponding to A) the penicillin sub-family, B) the cephalosporin sub-family, C) the carbapenem sub-family, and D) the monobactam family.

$\beta$ -lactam antibiotics inhibit the final step of bacterial cell wall synthesis. Specifically, they act as inhibitors for D-alanyl-D-alanine transpeptidases, more commonly referred to as the penicillin binding proteins, which are responsible for elongating and crosslinking the peptidoglycan in bacterial cell walls [4-6].  $\beta$ -lactam antibiotics mimic the structure of the D-alanyl-D-alanine crosslink in peptidoglycan thus acting as an irreversible inhibitor to the transpeptidase enzymes [7, 8]. Peptidoglycan is a cross-linked polymer that forms a net-like structure to support the cells' structure and protect the cell from the surrounding environment. Failure to form the peptidoglycan

structure results in growth inhibition, cell lysis, and ultimately cell death.  $\beta$ -lactam antibiotics can work on both gram-positive and gram-negative bacteria, but in gram-negative bacteria they must penetrate the cell membrane to get into the cell periplasm to access the penicillin-binding proteins [4]. Even though they are active against a wide range of bacterial pathogens, they have shown very little toxicity to mammalian cells, a fact which has contributed greatly to their success in clinical practice [2].

Over time, pathogens have developed resistance to  $\beta$ -lactam antibiotics in four ways [2, 4, 9]:

- 1.) Pathogenic strains produce  $\beta$ -lactamases, enzymes that catalyze the hydrolysis of the  $\beta$ -lactam ring and render the antibiotic ineffective.
- 2.) Pathogenic strains produce mutated penicillin-binding proteins to decrease their binding affinity for the  $\beta$ -lactam ring.
- 3.) Gram-negative pathogenic strains downregulate their porin channels to limit the entry of the antibiotic into the periplasmic space and thus block their access to the penicillin-binding proteins.
- 4.) Pathogenic strains are able to force the efflux of the antibiotic from the cell cytosol.

Perhaps the most drastic case of antibiotic resistance is the case of methicillin-resistant *Staphylococcus aureus*, which has developed resistance by way of downregulation of its porin channels. However, the most common reason for antibiotic resistance is the bacterial strains' production of  $\beta$ -lactamases [4, 6]. To date, there are more than 450 known  $\beta$ -lactamases.  $\beta$ -lactamases can be expressed in both gram-

negative and gram-positive bacteria and be either constitutively expressed or induced by exposure to  $\beta$ -lactam antibiotics [6]. In some cases, the effect of  $\beta$ -lactamases can be suppressed by administering a  $\beta$ -lactamase inhibitor along with the antibiotic. FDA approved inhibitors include sulbactam, tazobactam and clavulanate [10].

Despite the growing resistance to  $\beta$ -lactam antibiotics, their use in clinical practices to treat bacterial infections remains prevalent. In 2003 they comprised about 65% of the total world market for antibiotics, with cephalosporin dosage sales estimated to be \$9.9 billion and penicillin dosage sales estimated to be \$5 billion [11]. Furthermore, about half of the antibacterials listed on the World Health Organization's Model List of Essential Medicines are  $\beta$ -lactam antibiotics, with seven on the core list of medicines that are needed for a basic health care system and three on the complementary list of medicines that are needed for priority diseases. The core list medicines are considered to be the most safe, efficacious, and cost-effective medicines for current and future health care [12].

## **1.2 Synthesis Techniques for Semi-Synthetic $\beta$ -Lactam Antibiotics**

Only two  $\beta$ -lactam antibiotics that are used in clinical practice, penicillin G and penicillin V, are products of a fermentation process and thus considered to be naturally occurring molecules. The majority of  $\beta$ -lactam antibiotics used in clinical practices are classified as semi-synthetic because the  $\beta$ -lactam moiety is obtained from the enzymatic hydrolysis of a natural fermentation product; penicillin G, penicillin V, or cephalosporin C, while the acyl side chain is obtained by a chemical or chemoenzymatic synthesis.

### *1.2.1 Chemical Synthesis*

Chemical coupling of the  $\beta$ -lactam moieties with an acyl side chain has dominated the industrial production of semi-synthetic  $\beta$ -lactam antibiotics since their discovery in the early 1960s. The majority of semi-synthetic  $\beta$ -lactam antibiotics are still synthesized by way of the Dane anhydride process, which can achieve yields as high as 90% [13]. This process is carried out at temperatures as low as  $-30^{\circ}\text{C}$ , uses highly reactive pivaloyl chloride and silylating protection groups, and requires large volumes of dichloromethane, triethylamine and acetone for solvents. Despite recycling solvents and auxiliary reagents where possible, the Dane anhydride process still generates a large amount of non-biodegradable waste. Specifically, 30 kg to 40 kg of solid waste are generated for every kg of product in the synthesis of cephalexin [14].

### *1.2.2 Biocatalytic Synthesis*

The coupling of the  $\beta$ -lactam nuclei with the acyl side chain can be accomplished enzymatically utilizing penicillin G acylase (PGA). A less studied enzyme,  $\alpha$ -amino ester hydrolase (AEH), can also be used for this reaction when the acyl side chain has an amino group in the  $\alpha$ -position. Furthermore, the coupling can be carried out under thermodynamic control, which utilizes a non-activated acyl side chain, or kinetic control, which requires an activated side chain (typically an ester or amide). The thermodynamically-controlled and kinetically-controlled syntheses will be discussed in further detail in Chapter 2.

The first published example of the biocatalytic coupling of  $\beta$ -lactam antibiotics on an industrial scale was not until 1997 when DSM (Delft, Netherlands) opened a

production plant for cephalexin, marketed as Purilex®, in Barcelona, Spain. Initially it was reported that the penicillin acylase-catalyzed coupling reaction is performed in water between 0°C and 20°C with varying stoichiometric ratios of the nucleus and activated acyl side chain. The enzymatic coupling only achieved 40-60% conversion per cycle and had to be followed by several isolation and recycle steps [14]. Further reports indicate that the yields of the enzymatic coupling were able to be improved by using substrate concentrations as high as 0.3 to 0.5 M and using an excess of the activated acyl side chain [15]. DSM has since expanded their production of enzymatically synthesized  $\beta$ -lactam antibiotics under the umbrella name of DSMPureActives™ to also include amoxicillin, marketed as Purimox®, and cefadroxil, marketed as Puridrox® [16].

### **1.3 Comparison of Synthesis Techniques for Semi-Synthetic $\beta$ -Lactam Antibiotics**

The obvious disadvantage of the enzymatic coupling process in comparison to chemical coupling process is the lower yield of the product. However, the enzymatic coupling has undeniable advantages over the chemical coupling in terms of cost of raw materials, environmental impact, product quality, and ease of processing; due to the fact that it is carried out at ambient temperature, pressure and pH; and does not require toxic or hazardous reagents or solvents [13]. From an environmental standpoint, by switching from the chemical coupling to the enzymatic coupling, DSM has reduced process greenhouse emissions by over 50%, reduced process emissions to water by 50%, and reduced process waste by 90%. From a product quality standpoint, it has increased the average purity by 1.6%, 1.4%, and 1.2% as assayed “on dry” high performance liquid chromatography for Purimox®, Purilex® and Puridrox®, respectively. For an example



of the impurities eliminated by switching to the biocatalytic coupling in the production of cefadroxil, see Table 1.1. Furthermore, they report an increased shelf life of the enzymatically synthesized products and better tasting end products for patients. From a processing standpoint, DSM has reduced the number of processing steps by 50% and increased the size of batch production by at least 3-fold and as much as 10-fold. The decrease in number of impurities and increase in the size of batch production translates into a significant reduction in the amount of quality control testing that is required for all pharmaceutical products [16].

Table 1.1: Comparison of impurities present in the enzymatically-coupled antibiotic Puridrox® and the industrial average for chemically-coupled cefadroxil [16].

<b>Impurity</b>	<b>Impurity Source</b>	<b>Puridrox®</b>	<b>Industry Average</b>
acetone (%)	solvent	< 0.01	0.01-0.08
methylene chloride (ppm)	solvent	NP	1.5-100
pivalic acid (ppm)	solvent	NP	<10
dimethylformamide (%)	solvent	NP	0.0-0.37
( <i>R</i> )-hydroxyphenylglycine	undesired product	0.03-0.07	0.04
7-aminodesacetoxycephalosporanic Acid	reactant	0.09-0.16	0.08

## 1.4 Biocatalysts Used to Synthesize $\beta$ -Lactam Antibiotics

### 1.4.1 Penicillin G Acylases

As discussed previously, PGA (EC 3.5.1.11) is a very important enzyme in the industrial production of semi-synthetic  $\beta$ -lactam antibiotics, as it is used to obtain the  $\beta$ -lactam nuclei from naturally occurring antibiotics and can be used for the coupling of the  $\beta$ -lactam nuclei to acyl side chains. Discovered in 1960, PGAs are thought to be used to

generate carbon sources from aromatic compounds *in vivo* [15, 17]. Bacteria, actinomycetes, yeasts, and fungi all produce PGA [18]. This discussion will be focused on the PGA strain ATCC 11105 from *Escherichia coli* because it is the most studied of the PGAs [15].

#### 1.4.1.1 Structure and Catalytic Mechanism of Penicillin G Acylases

Mature PGA is a heterodimeric enzyme with a 23.9 kDa  $\alpha$ -chain and a 62.4 kDa  $\beta$ -chain [19-21]. The enzyme's two chains are closely intertwined and form a 70 Å x 50 Å x 55 Å protein that is kidney-shaped in its cross section [19, 21] and has a cone-shaped depression in the center of the protein surface, at the base of which lies the catalytic residue. PGA belongs to the N-terminal nucleophile (Ntn) hydrolase superfamily, which is characterized by a single N-terminal catalytic residue and a characteristic four layer  $\alpha + \beta$  structure around the active site [19, 20]. Refer to Figure 1.2 for the crystal structure of mature PGA.



Figure 1.2: The subunit-colored crystal structures of PGA from *E. coli* (PDB 1GK9).

Like all Ntn hydrolases, PGA is expressed as an inactive precursor peptide that must undergo post-translational processing to render the mature form [20]. Specifically, PGA is expressed as a single cytoplasmic precursor which contains an N-terminal 26 amino acid signal sequence, a 209 amino acid  $\alpha$ -chain, a 54 amino acid spacer sequence, and a 557 amino acid  $\beta$ -chain. The signal sequence is responsible for the protein's transport from the cytoplasm and is cleaved upon the protein's arrival into the periplasm. The linker sequence aids in the protein's folding and is subsequently cleaved to expose the active residue. Both the sequence and linker cleavage processes are believed to be autocatalytic [19, 20, 22]. Interestingly, a precursor peptide mutated to undergo slow post-translational processing has shown that the periplasmic precursor protein has an almost identical fold to mature PGA, the two forms have only a 0.71 Å r.m.s. difference between the alpha carbons in their  $\alpha$  and  $\beta$  chains. The periplasmic precursor protein crystal structure shows that the linker peptide is compactly packed against the surface depression and the active site observed in mature PGA [22].

The catalytic mechanism of PGA has been elucidated through crystal structures and mutagenesis studies. PGA has a hydrophobic binding pocket lined with aromatic and hydrophobic residues that is specific for phenylacetyl groups and their derivatives [19-21]. The range of substrate specificity and enantioselectivity of PGA for phenylacetyl derivatives is determined by the size and polarity of their substituents [23]. Residues in the active site undergo a conformational shift upon substrate binding to create a binding site for the penicillin nucleus, therefore, PGA substrate binding is an induced fit mechanism [19, 20, 24, 25]. Bound substrates are in the proper position for the catalytic serine, located at the  $\beta$ 1 position, to perform a nucleophilic attack on the carbonyl carbon

of the substrate to form an oxyanion tetrahedral transition state and subsequently a acyl-enzyme intermediate [21]. A nucleophile, which can be a  $\beta$ -lactam nucleus for a coupling reaction, or water for a hydrolysis reaction, then deacylates the acyl-enzyme intermediate to form products. PGA's residues that are important for catalysis and their respective functions are listed in Table 1.2. As shown in Table 1.2, mutation of the active site residues frequently leads to a buildup of the inactive precursor in the periplasm, indicating that the residues that participate in catalysis also participate in the autocatalytic cleavage of the linker peptide [19, 24, 25]. Crystal structures of a PGA mutant undergoing slow post-translational processing agree with this fact [22].

#### 1.4.1.2 Kinetic Parameters of Penicillin G Acylases

The kinetic parameters of PGA are shown in Table 1.3. Ideally, the activity of PGA for the  $\beta$ -lactam antibiotic would be much lower in comparison to the activated acyl side chains so as to enhance the rate of the coupling reaction and decrease the rate of the hydrolysis reactions that occur in the kinetically-controlled synthesis of  $\beta$ -lactam antibiotics. However, the kinetic parameters reported show that instead  $k_{\text{cat}}/K_m$  values of the antibiotic are about 10 times higher than that of the activated acyl side chains [26, 27].

Table 1.2: Important residues for catalysis in PGA from *E. coli*.

Residue	Function	Source
$\alpha$ Met142	Interacts with phenylacetyl side-chain of substrate.	[19]
$\alpha$ Arg145	Able to have two different conformations: 1)'Closed' helical conformation: no substrate is bound; residue side-chain is hydrogen bonded to $\beta$ Phe24 2) 'Open' coil conformation: bound substrate; residue side-chain interacts with carboxylate oxygen of $\beta$ -lactam nucleus via two bridging water molecules.  Mutation to Leu, Cys, or Lys results in correctly processed mutant but a 5-50 fold reduction in $k_{cat}/K_m$ for the chromogenic reference substrate 2-nitro-5-[(phenylacetyl)amino]-benzoic acid.	[19, 20, 24-26]
$\alpha$ Phe146	Able to have two different conformations: 1)'Closed' helical conformation: no substrate is bound; residue closes hydrophobic active site from solvent 2) 'Open' coil conformation: bound substrate; side-chain interacts with $\beta$ -lactam nucleus.  Structurally linked to $\beta$ Phe71 via a calcium ion.  Limits the size of the $C_\alpha$ -substituent that can bind in phenylacetic acid derivatives.	[19, 20, 23, 24]
$\beta$ Ser1	Acts as catalytic residue by performing nucleophilic attack on substrate's carbonyl group to form oxyanion tetrahedral transition state.  In proper position to perform nucleophilic attack that cleaves linker peptide.  Mutation to an Ala results in accumulation of precursor protein.	[19, 21, 22]
$\beta$ Gln23	Mutation to a Cys results correct processing but Cys is susceptible to oxidation, which results in poor substrate binding. Main chain amide stabilizes tetrahedral intermediate by forming part of oxyanion hole.  Main chain oxygen might have van der Waals interactions with carboxyl oxygen of $\beta$ -lactam ring.  Main chain amide thought to be in proper position to stabilize tetrahedral transition state in linker cleavage reaction by forming part of oxyanion hole.	[19, 21, 22, 24]

Table 1.2 continued: Important residues for catalysis in PGA from *E. coli*.

Residue	Function	Source
$\beta$ Phe24	Interacts with phenylacetyl side-chain of substrate.  Limits the size of the C $_{\alpha}$ -substituent that can bind in phenylacetic acid derivatives.	[19, 23]
$\beta$ Ser67	Side chain interacts with hydroxyl group in <i>p</i> -hydroxybenzylpenicillin and increases the rate of hydrolysis (relative to benzylpenicillin)	[21]
$\beta$ Ala69	Main chain amide stabilizes tetrahedral intermediate by forming part of oxyanion hole.  Main chain amide thought to be in proper position to stabilize tetrahedral transition state in linker cleavage reaction by forming part of oxyanion hole.	[19-22, 24, 26]
$\beta$ Phe71	Slightly changes conformation upon binding with substrate to form a stacked conformation with thiazolidine ring of $\beta$ -lactam nucleus.  Structurally linked to $\alpha$ Phe146 via a calcium ion.	[19, 24]
$\beta$ Ile177	Interacts with phenylacetyl side-chain of substrate.	[19]
$\beta$ Asn241	N $\delta$ stabilizes tetrahedral transition state by forming part of oxyanion hole.  N $\delta$ thought to be in proper position to stabilize tetrahedral transition state in linker cleavage reaction by forming part of oxyanion hole.  Aides in positioning bound substrate, especially the appropriate placement of the amide bond for catalysis.	[19-22, 24, 26]
$\beta$ Asn263	Mutation to an Ala results mix of processed and precursor protein. Residue must be protonated for catalysis to occur as the charge effects the electrostatic environment of oxyanion hole and could possibly affect transition state stabilization.  Interacts with $\beta$ -lactam nucleus of the substrate through salt bridge.  Mutation to an Ile, Leu or Asn results in accumulation of precursor protein.  Mutation to a Lys results in partially processed precursor protein, as evidenced by separate $\alpha$ and $\beta$ chains on SDS/PAGE gel.	[19, 25]

Table 1.3: Selected kinetic parameters for PGA from *E. coli*.

Substrate	$K_m$ (mM)	$k_{cat}$ (s <sup>-1</sup> )	$k_{cat}/K_m$ (s <sup>-1</sup> mM <sup>-1</sup> )	Temperature (°C)	pH	Source
( <i>R</i> )-phenylglycine amide	27.4	35.6	1.3	30	7.0	[28, 29]
( <i>R</i> )-phenylglycine methyl ester	12.5	50	4	30	7.0	[27]
ampicillin	3.6	36.7	10.19	30	7.0	[28, 29]
cephalexin	1.5	29	19.3	30	7.0	[30]
amoxicillin	1.07	17	15.9	30	7.0	[30]
cefadroxil	0.642	32	49.8	30	7.0	[30]
penicillin G	0.013	39	3000	30	7.0	[30]

#### 1.4.1.3 Protein Engineering of Penicillin G Acylases

The majority of protein engineering of PGA has targeted increasing the synthesis of the antibiotic and decreasing the primary hydrolysis of the activated acyl side chain and the secondary hydrolysis of the antibiotic in the biocatalytic coupling of the  $\beta$ -lactam nucleus and the activated acyl side chain. Alkema et al. and Jager et al. have attempted this feat with both rational and combinatorial design but have had limited success [25, 26, 28, 29]. Alkema et al. examined the role of residue  $\alpha$ Arg145 and  $\beta$ Arg263 using site-directed mutagenesis (the amino acid residues are labeled to indicate the chain,  $\alpha$  or  $\beta$ , the wild-type amino acid residue, the residue number on the chain, and the variant amino acid residue when applicable).  $\alpha$ Arg145 variants showed an increase in affinity for the  $\beta$ -lactam nucleus as a nucleophile compared to water at the expense of significantly decreased activity.  $\beta$ Arg263 was determined to be important for precursor processing but not involved in substrate binding [25]. Jager et al. examined the importance of  $\alpha$ Arg145 and  $\alpha$ Phe146 on PGA nucleophile selectivity via site-saturation mutagenesis. These residues were chosen based on the fact that crystal structures show that they

undergo a 3.5 Å conformational shift towards the solvent upon ligand binding. Both sets of variants had improved synthesis to hydrolysis ratios but again at the expense of activity. For example the  $\alpha$ Arg145Trp variant had synthesis to hydrolysis ratio of 14.3, nearly a 10-fold increase over the wild-type enzyme, but showed only 6% of the wild-type PGA activity [29].

Protein engineering on PGAs has also been employed to improve its thermostability. Polizzi et al. used the structure-guided consensus concept to target potentially thermostabilizing mutations on 20 residues. Of these variants, 50% had an increased half-life at 50°C, 10% had a half life equivalent to that of the wild-type enzyme, 20% had a decreased half-life and the remaining variants' activity was too low to determine an accurate half-life. Variants  $\alpha$ Trp25Tyr and  $\alpha$ Ala80Arg improved the half-life of PGA by about 2.5 and the activity by about 1.5 in comparison to wild-type PGA [31].

#### *1.4.2 $\alpha$ -Amino Ester Hydrolases*

AEHs (E.C. 3.1.1.43), also referred to as ampicillin acylases, are a family of enzymes that are interesting because they are capable of the synthesis and hydrolysis of semi-synthetic antibiotics [32-36]. In other words, they are alternatives to PGAs. As their name implies, AEHs are highly specific for substrates which contain an  $\alpha$ -amino substituent, such as ampicillin and cephalexin. In fact, they have been shown to hydrolyze  $\beta$ -lactam antibiotics containing an  $\alpha$ -amino group, with a twofold higher activity compared to penicillin G [37]. Their unique substrate specificity renders them interesting candidates for the enzymatic one-pot synthesis of ampicillin, as the penicillin



G hydrolysis product, phenylacetic acid, does not inhibit them. Discovered by Takahashi in 1972, recombinant AEHs have been isolated and characterized from *Acetobacter turbidans*, *Xanthomonas citri*, and *Xanthomonas campestris* pv. *campestris* [34-36]. Several other proteins that have the same substrate range as AEHs have been isolated from their wild-type organisms including a cephalixin-synthesizing enzyme from *Gluconobacter oxydans*, and novel PGAs from *Achromobacter* sp. CCM 4824 [38, 39].

#### 1.4.2.1 Structure and Catalytic Mechanism $\alpha$ -Amino Ester Hydrolases

The crystal structures for AEH from both *A. turbidans* and *X. citri* have been solved. As shown in Figure 1.3, the protein is a homotetramer with 72 kDa subunits, totaling 288 kDa [34, 35]. The ligand binding site is on the interior of the homotetramer and must pass through one of two 15 Å wide entrances to reach the binding region [34]. The enzymes belong to the subgroup of serine hydrolases from the  $\alpha/\beta$ -hydrolase fold family. Hence, the enzyme has a Ser-His-Asp catalytic triad and an oxyanion hole.

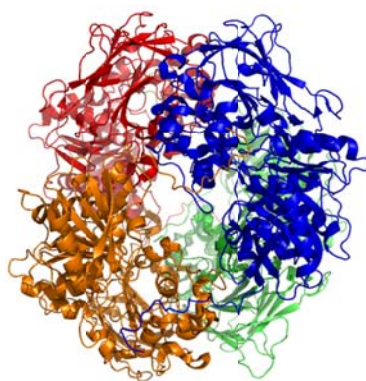


Figure 1.3: The subunit-colored crystal structure of AEH from *X. citri* (PDB 2B4K).

AEHs are unique in that an acidic cluster, Asp-Glu-Asp, termed a carboxylate cluster, has been identified in the active site of the protein. This cluster is associated with the enzyme's unique  $\alpha$ -amino substrate specificity [34, 40, 41]. AEHs are very substrate-specific for  $\alpha$ -amino acid derivatives, such as ampicillin, but can accept the cephalosporin  $\beta$ -lactam nuclei 7-aminocephalosporanic acid (7-ACA) and 7-aminodeacetoxycephalosporanic acid (7-ADCA), or the penicillin  $\beta$ -lactam nucleus 6-aminopenicillanic acid (6-APA) with some preference for 7-ADCA. The enzyme has no detected activity for penicillin G which lacks an  $\alpha$ -amino substituent [33, 41-43].

#### 1.4.2.2 Kinetic Parameters of $\alpha$ -Amino Ester Hydrolases

AEHs were evaluated for use in the synthesis of the cephalosporins cephalexin and cephaloglycine from (*R*)-phenylglycine methyl ester ((*R*)-PGME) and 7-ACA or 7-ADCA, respectively [32, 33]. The kinetic parameters for AEHs are shown in Table 1.4. Several organisms were used in Takahashi's studies, however, the highest product concentrations were obtained from the strains IFO 3835 from *X. citri* and ATCC 9325 from *A. turbidans* [32, 33, 42].

#### 1.4.2.3 Protein Engineering of $\alpha$ -Amino Ester Hydrolases

The synthesis parameters have been reported in the synthesis of cephalexin from 7-ADCA and (*R*)-PGME. Conversions as high as 75% have been reported with a non-recombinant preparation of AEH from *X. citri* under optimized conditions [44]. Barends reported that mutating the tyrosine adjacent to the catalytic serine to alanine, Tyr206Ala, improved its initial synthesis to hydrolysis ratio and improved its maximum product yield

from 36% to 58% conversion in a reaction containing 15 mM (R)-PGME and 30 mM 6-APA [35]. However, there is still significant room for improvement on these enzymes' synthesis parameters. Very little work has been done on optimizing the reaction conditions and very little protein engineering has been attempted with the AEHs.

Table 1.4. Selected kinetic parameters for AEHs.

Substrate	$K_m$ (mM)	$k_{cat}$ (s <sup>-1</sup> )	$k_{cat}/K_m$ (s <sup>-1</sup> mM <sup>-1</sup> )	Temperature (°C)	pH	Source
<u><i>A. turbidans</i></u>						
(R)-phenylglycine amide	>13	>43	3.3	30	6.0	[40]
(R)-phenylglycine methyl ester	7	1035	148	30	6.0	[40]
ampicillin	1.0	162	162	30	6.0	[40]
cephalexin	0.34	347	1021	30	6.0	[40]
amoxicillin	2.6	10	3.9	30	6.0	[40]
cefadroxil	1.7	9.6	6	30	6.0	[40]
<u><i>X. citri</i></u>						
(R)-phenylglycine amide	ND	1.6	-	25	6.4	[34]
(R)-phenylglycine methyl ester	90	1860	21	25	6.4	[34]
ampicillin	1.2	58	48	25	6.4	[34]
cephalexin	1.8	160	89	25	6.4	[34]
amoxicillin	-	-	2	25	6.4	[34]
cefadroxil	-	ND	-	25	6.4	[34]
penicillin G	-	ND	-	25	6.4	[34]
<u><i>X. campestris</i> pv. <i>campestris</i></u>						
ampicillin	1.1	72.5	64.5	25	6.8	[45]
cephalexin	2.2	200	95	25	6.8	[45]
penicillin G	-	ND	-	25	6.8	[45]

## 1.5 Research Objectives and Organization

The overarching goal of the research presented in this dissertation is to investigate methods that will lead to an improvement in the enzymatic synthesis of semi-synthetic  $\beta$ -lactam antibiotics. Specifically, we sought to improve the enzymatic synthesis of semi-synthetic  $\beta$ -lactam antibiotics with three different approaches: reaction engineering, medium engineering, and data-driven protein engineering. Our studies primarily focus on the penicillin G acylase-catalyzed kinetically-controlled synthesis of ampicillin from the acyl side chain (*R*)-phenylglycine methyl ester and the  $\beta$ -lactam nucleus 6-aminopenicillanic acid, though some variations in the catalyst and acyl side chain donors utilized will be discussed. Though focused on ampicillin synthesis, the research results presented could be applicable to the synthesis of amoxicillin, cephalexin, cefadroxil, cefaclor, and cefprozil.

### *1.5.1 Improving the Enzymatic Synthesis of Semi-Synthetic $\beta$ -Lactam Antibiotics via Reaction Engineering*

Chapter 2 of this dissertation will begin with a discussion of the thermodynamically-controlled and kinetically-controlled enzymatic syntheses of semi-synthetic  $\beta$ -lactam antibiotics. Subsequently, the feasibility of utilizing a cascade conversion involving two biocatalytic steps in fully aqueous medium to produce ampicillin will be presented. The cascade conversion seeks to combine the production of the  $\beta$ -lactam nucleus 6-APA from penicillin G utilizing immobilized PGA and the synthesis of ampicillin from 6-APA and (*R*)-PGME utilizing either AEH or immobilized PGA. This set-up presents a promising alternative to the current two-pot set-up which requires intermittent isolation of the intermediate, 6-APA.

In this work, two different reaction configurations and various relative enzyme loadings were studied. In a one-pot, one-step configuration, a batch process, penicillin G, (R)-PGME, and all enzyme(s) were added to the reaction and the reaction was allowed to proceed. In a one-pot, two-step configuration, penicillin G and PGA were added to the reaction and the hydrolysis reaction was allowed to proceed. When the hydrolysis reaction was near completion, (R)-PGME and either AEH or PGA was added to the reaction. The choice of enzymes and the effect of relative enzyme loadings for each set-up will be presented, and the disadvantages and benefits of each set-up will be discussed.

#### *1.5.2 Improving the Enzymatic Synthesis of Semi-Synthetic $\beta$ -Lactam Antibiotics via Medium Engineering*

Initially, the synthesis of ampicillin in organic co-solvents was chosen for investigation due to the known advantages of enzymatic syntheses in organic co-solvents which include increased solubilities of reactants and products, shifting reaction equilibria, and most important for the enzymatic coupling step in  $\beta$ -lactam antibiotic synthesis, suppressing hydrolysis reactions. Ethylene glycol was chosen as a starting point after a review of the literature. In Chapter 3 of this thesis, the effects of performing the synthesis of ampicillin in the presence of an ethylene glycol co-solvent will be presented.

Interestingly, it was determined that the benefit of utilizing ethylene glycol as a co-solvent varies for different starting ratios of 6-APA to (R)-PGME. Additionally, it was determined that the transesterification product of (R)-PGME and ethylene glycol, (R)-phenylglycine hydroxyethyl ester ((R)-PGHEE), was forming during the synthesis reactions. In contrast, the formation of (R)-PGHEE was not observed in the synthesis of ampicillin from 6-APA and (R)-phenylglycine amide. In this work, the results for

synthesis of ampicillin in ethylene glycol from 6-APA and different acyl side chains will be compared in order to demonstrate the effects of (R)-PGHEE formation on the overall yield observed in the kinetically-controlled synthesis of ampicillin.

### *1.5.3 Improving the Enzymatic Synthesis of Semi-Synthetic $\beta$ -Lactam Antibiotics via Data-Driven Protein Engineering*

Chapter 4 of this dissertation will discuss how data-driven protein engineering was employed to improve the diastereomeric selectivity of PGA. Specifically, the selectivity of PGA with respect to the amino group on the alpha carbon in the synthesis of ampicillin from racemic phenylglycine methyl ester (*rac*-PGME) and 6-APA was targeted for improvement. Currently, when starting with *rac*-PGME, PGA is only weakly selective for the desired product, (*R*)-ampicillin, over the undesired product, (*S*)-ampicillin. Development of a selective PGA would enable the use of racemic acid derivatives in  $\beta$ -lactam antibiotic synthesis.

In order to alter the selectivity of PGA, five residues in the active site were chosen for randomization. All five libraries were screened and positive hits were subsequently sequenced and characterized in reactions with *rac*-PGME and pure PGME enantiomers. The explanation of improved selectivity properties observed in PGA variants will be presented based on existing knowledge of PGA crystal structures and mutagenesis studies.

## **1.6 Publication Information**

The review of  $\beta$ -lactam antibiotics, the associated synthesis techniques, and biocatalysts involved is published in Wiley's Encyclopedia of Industrial Biotechnology

(Ed. M.C. Flickinger, 2010, pp 535-567) with Janna K. Blum and Andreas S. Bommarius  
as co-authors.

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## CHAPTER 2

### IMPROVING THE ENZYMATIC SYNTHESIS OF $\beta$ -LACTAM ANTIBIOTICS VIA REACTION ENGINEERING

#### 2.1 Introduction

##### 2.1.1 *Enzymatic Synthesis of Semi-Synthetic $\beta$ -Lactam Antibiotics*

Semi-synthetic  $\beta$ -lactam antibiotics are classified as such because their  $\beta$ -lactam moiety is obtained from a natural fermentation product and their acyl side chain is obtained from a chemical or chemoenzymatic synthesis. As mentioned in Chapter 1, the enzymatic coupling of an acyl side chain with a  $\beta$ -lactam nucleus can be accomplished utilizing either penicillin G acylase (PGA) or  $\alpha$ -amino ester hydrolase (AEH). Regardless of the biocatalyst chosen, the synthesis of  $\beta$ -lactam antibiotics can be carried out under thermodynamic control, which utilizes a non-activated acyl side chain, or kinetic control, which requires an activated side chain (typically an ester or amide). See Figure 2.1 for a comparison of the thermodynamic and kinetic enzymatic syntheses of ampicillin.

The thermodynamically-controlled synthesis, also referred to as the direct or equilibrium-controlled synthesis, is only able to achieve minimal yields (1% or less) in aqueous medium, as the equilibrium lies very far to the left [1-4]. At typical reaction conditions the substrates are in their ionized forms, which PGA does not accept [2, 4]. Therefore, the thermodynamically-controlled enzymatic synthesis usually has very slow rates in addition to very low yields. Adding organic co-solvents to decrease the water activity and thus shift the equilibrium towards the antibiotic has been studied with limited

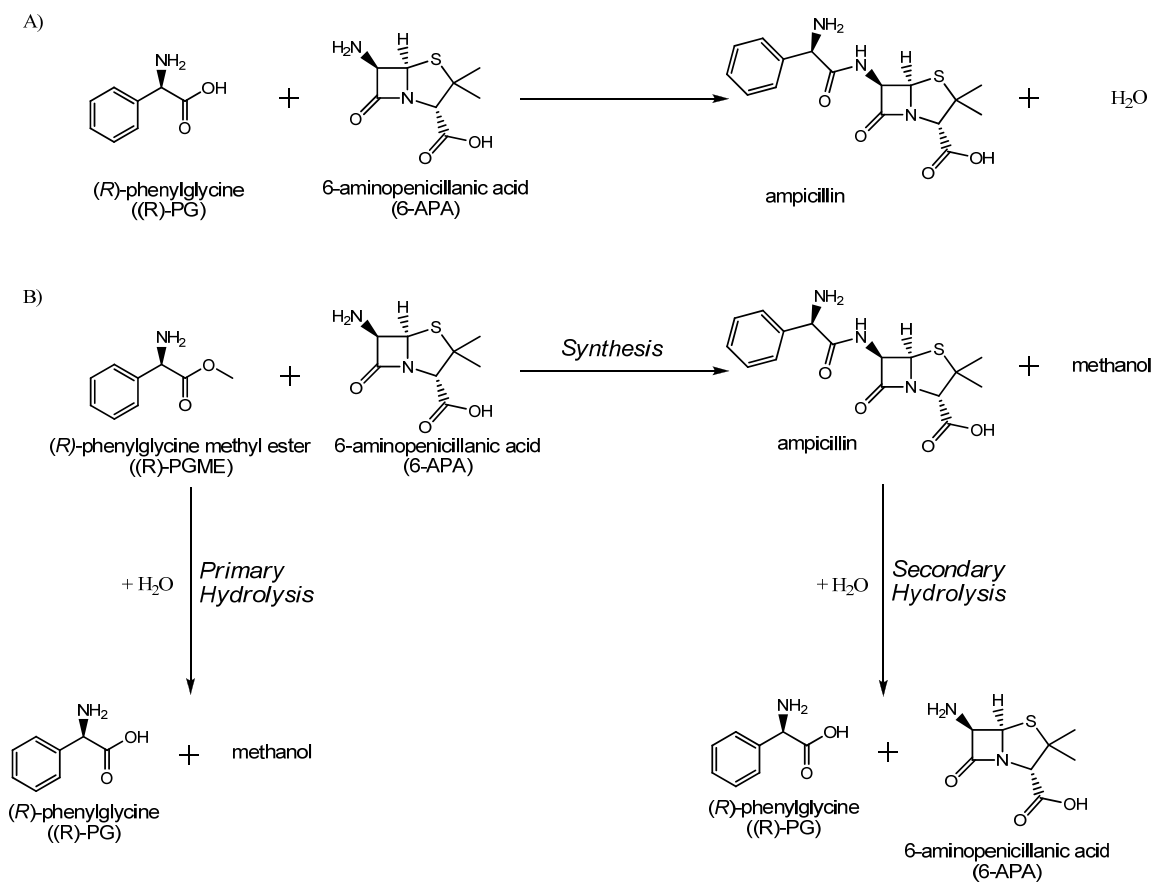


Figure 2.1: The PGA catalyzed coupling of ampicillin via A) a thermodynamically-controlled route starting from (R)-phenylglycine ((R)-PG) and 6-aminopenicillanic acid (6-APA) and B) a kinetically-controlled route starting from (R)-phenylglycine methyl ester ((R)-PGME) and 6-APA.

success [1-4]. For example, in the case of cephalexin synthesis, the yield was only improved from 0.75% in aqueous medium to 1.25% in 36% (v/v) triglyme [4].

Though the thermodynamically-controlled system is not useful for the synthesis of semi-synthetic  $\beta$ -lactam antibiotics, the equilibrium is taken advantage of to synthesize the  $\beta$ -lactam nucleus 6-aminopenicillanic acid (6-APA) though the hydrolysis of the naturally occurring penicillin G using PGA or penicillin V using penicillin V acylase (EC 3.5.1.11). 6-APA is the  $\beta$ -lactam nucleus used to synthesize all semi-synthetic penicillins. Furthermore, its five-membered ring can be expanded to a 6-membered ring to generate 7-aminodesacetoxycephalosporanic acid (7-ADCA), the  $\beta$ -lactam nucleus for a number of cephalosporins [5]. In 2003, 6-APA was the world's largest selling  $\beta$ -lactam intermediate with a worldwide production of 8,800 tons per year [5, 6].

Typically, the kinetically-controlled synthesis is employed for the coupling of the  $\beta$ -lactam nuclei and the acyl side chain. However, the kinetically-controlled reaction results in low yields (between 30 and 40%) because PGA catalyzes the undesired primary hydrolysis of the activated acyl side chain donor and the secondary hydrolysis of the antibiotic in addition to the desired synthesis reaction (again, refer to Figure 2.1). This phenomenon can be explained by the specificity and catalytic mechanism of PGA described in Chapter 1. As PGA is specific for only the phenylacetyl portion of its substrates, acyl side chains and  $\beta$ -lactam antibiotics both bind to the enzyme. These electrophilic substrates are attacked by the catalytic serine of PGA and an acyl-enzyme intermediate is formed. Subsequently a nucleophile, which can be a  $\beta$ -lactam nucleus for a coupling reaction or water for a hydrolysis reaction, deacylates the acyl-enzyme intermediate to form products.

### 2.1.2 One-Pot Synthesis of Semi-Synthetic $\beta$ -Lactam Antibiotics

Cascade conversions, which combine multiple reactions without intermediate recovery steps, are increasingly studied to render syntheses more environmentally benign and economically advantageous. Replacing a multi-step synthesis requiring intermittent isolation with a cascade process eliminates the need for isolation and purification of intermediates and therefore results in smaller reactor volumes, shorter cycle times, higher volumetric and space time yields, and decreased amount of waste produced [7, 8]. Cascade conversions can combine multiple biocatalytic steps, multiple chemocatalytic steps, or can combine both biocatalytic and chemocatalytic steps. Typically it is easiest to combine multiple biocatalytic steps as most enzymes have similar operating conditions [7]. There have been several reports of utilizing cascade processes for semi-synthetic  $\beta$ -lactam antibiotic synthesis. Wegman et al. combined the synthesis of the acyl side chain (*R*)-phenylglycine amide from (*R*)-phenylglycine nitrile utilizing nitrile hydratase and the enzymatic coupling of (*R*)-phenylglycine amide with the  $\beta$ -lactam nucleus 7-ADCA utilizing PGA to synthesize cephalexin in a one-pot synthesis[9]. Fernández-Lafuente et al. reported a chemoenzymatic synthesis of cefazolin that started from the naturally occurring cephalosporin C and involved three biocatalytic transformations in fully aqueous medium [10, 11]. Finally, Du et al. and Wu et al. employed PGA in partially organic media to catalyze both the hydrolysis of penicillin G to the  $\beta$ -lactam nucleus 6-APA and the enzymatic coupling of 6-APA with (*R*)-phenylglycine methyl ester ((*R*)-PGME) or (*R*)-hydroxyphenylglycine methyl ester to synthesize ampicillin or amoxicillin, respectively, in a one-pot system [12, 13].

In this work, we examined the feasibility of utilizing a cascade conversion with two biocatalytic reactions in fully aqueous medium to synthesize ampicillin as shown in Figure 2.2. In the first reaction, 6-APA was produced from the thermodynamically-controlled hydrolysis of penicillin G with iPGA. The by-product from this reaction, phenylacetic acid (PAA), is a known inhibitor of PGA with an inhibition constant equal to 70  $\mu$ M [14]. In the second reaction, ampicillin was produced in a kinetically-controlled coupling of 6-APA and (R)-PGME with either iPGA or AEH [15]. As AEHs are unique in their specificity toward  $\alpha$ -amino groups on the acyl moiety, they cannot catalyze the hydrolysis of penicillin G to yield 6-APA and are not inhibited by PAA, thus their advantage in this cascade [16].

We investigated both a one-pot, one-step (1P1S) and one-pot, two-step (1P2S) scheme. In the 1P1S scheme, a batch process, we added (R)-PGME, penicillin G, and either iPGA or both iPGA and AEH at the beginning of the experiment. In the 1P2S scheme, we first added penG with iPGA and allowed the reaction to proceed near completion to produce 6-APA. Next, we added (R)-PGME and either AEH or additional iPGA to the reaction mixture. We investigated the effect of different relative enzyme loadings on the overall yield of ampicillin for both schemes.



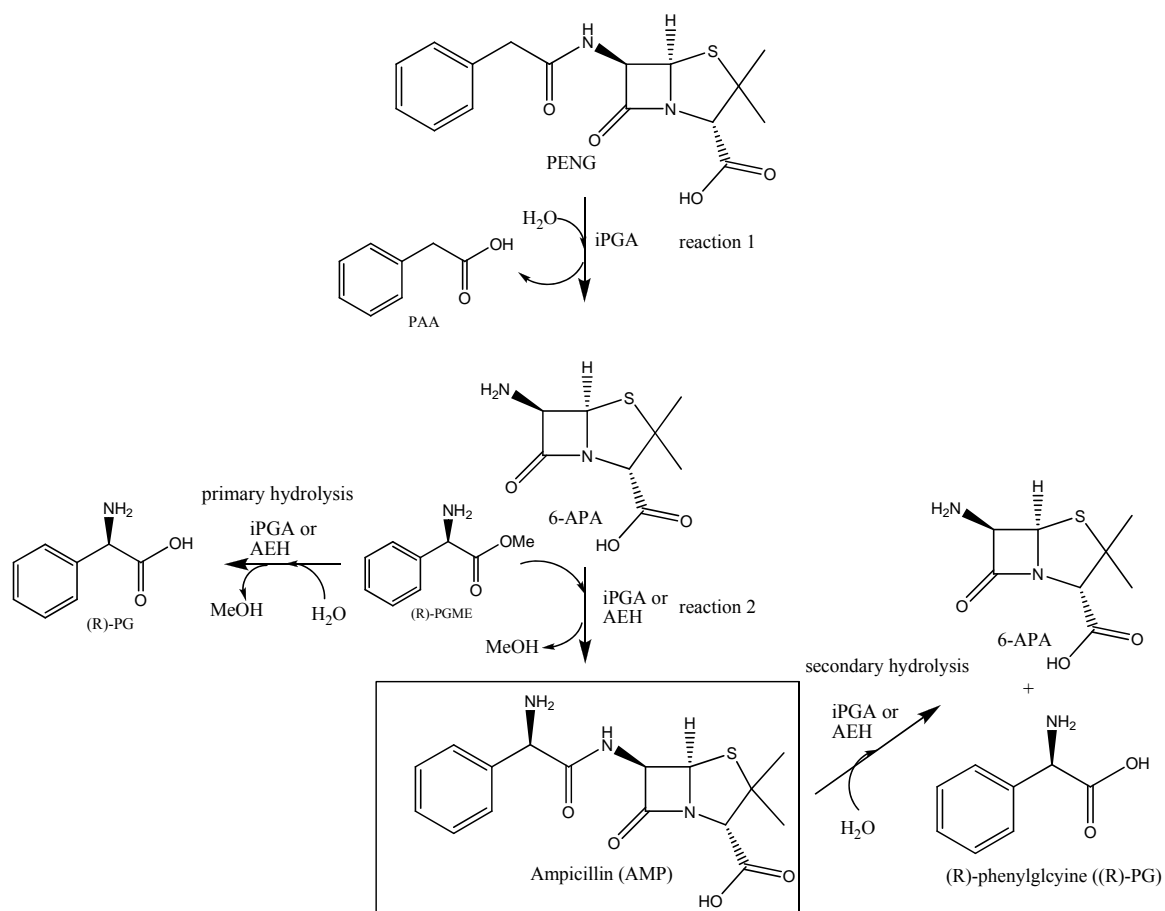


Figure 2.2: One-pot, two-enzyme direct conversion of penicillin G (PENG) to ampicillin (AMP) using iPGA and AEH. Undesired side reactions, primary hydrolysis of (R)-PGME and secondary hydrolysis of ampicillin, are shown.

## 2.2 Materials and Methods

### 2.2.1 Materials

6-Aminopenicillanic acid (6-APA), (R)-phenylglycine ((R)-PG), ampicillin, (R)-phenylglycine methyl ester hydrochloride ((R)-PGME), penicillin G, phenylacetic acid (PAA), and Eupergit<sup>®</sup>-immobilized penicillin G acylase from *Escherichia coli* all were procured from Sigma Aldrich (St. Louis, MO, USA). Soluble amino ester hydrolase from *Xanthomonas campestris pv. campestris* was prepared as described in Blum et al, 2010 [15] .

### 2.2.2 Ampicillin Synthesis with Immobilized Penicillin G Acylase

10 mL reactions with 20 mM 6-APA and 60 mM (R)-PGME in 100 mM phosphate buffer at pH 7 and room temperature (22°C-25°C) were carried out with 250 mg iPGA, 124 UPenG per gram of carrier, where 1 UPenG is defined as one  $\mu$ mol of penicillin G hydrolyzed per minute.

### 2.2.3 One-Pot, One-Step Synthesis

15 mL of 20 mM of penicillin G and 60 mM (R)-PGME in 100 mM phosphate buffer at pH 7 were added to a round bottom flask along with iPGA or iPGA and purified *X. campestris pv. campestris* AEH, per Table 2.1. The reactions were stirred using a magnetic stir plate and carried out at room temperature (22°C-25°C).

#### 2.2.4 One-Pot, Two-Step Synthesis

7.5 mL of 40 mM penicillin G in 100 mM phosphate buffer at pH 7 was added to a round bottom flask along with iPGA per Table 2.2, 124 UPenG per gram of carrier, where 1 UPenG is defined as one  $\mu\text{mol}$  of penicillin G hydrolyzed per minute. The reactions were stirred using a magnetic stir plate and carried out at room temperature (22°C-25°C). After the reaction reached near completion, as determined by high performance liquid chromatography (HPLC), 7.5 mL of 120 mM (R)-PGME was added. The pH was adjusted with sodium hydroxide from  $\sim 6.4$  to 7.0 and *X. campestris pv. campestris* AEH was added per Table 2.2, 79 UAmp per mg protein, where UAmp is defined as one  $\mu\text{mol}$  of ampicillin hydrolyzed per minute under saturation conditions. In reactions where iPGA was used in both steps, we replaced the AEH with equivalent ampicillin synthesis units of iPGA based on initial synthesis rate data from 6-APA and (R)-PGME using only AEH and only iPGA where 1 UAmp of AEH  $\approx$  1UAmp of iPGA  $\approx$  6.8 UPenG of iPGA [15].

#### 2.2.5 One-Pot, Two-Step, Two-Stage Synthesis

These experiments were conducted analogously to the 1P2S schemes, with the exception that after the completion of the first step the iPGA was removed from the reaction using filtration.

#### 2.2.6 High Performance Liquid Chromatography Assay

All analysis was conducted using HPLC complete with a Shimadzu-LC-20AT pump, Beckman Coulter Ultrasphere ODS 4.6 mm x 25 cm column, and SPD-M20A

prominence diode array detector monitored at 215 nm. Samples were diluted 10X into HPLC quench buffer composed of 75% Methanol, 25% 0.02 M potassium phosphate, pH 6.0. 2  $\mu$ L of sample was loaded onto the column. The mobile phase operated under a gradient at 1.0 mL/min and contains 20% methanol and 80% 0.02 phosphate buffer at pH 7 and increased to 50% methanol over a 25 min period. All components, (R)-PG, PAA, 6-APA, (R)-PGME, ampicillin and penicillin G were detected using this method. Results were normalized based on the penicillanic ring mass balance. More details on the HPLC method utilized can be found in Appendix B.

## **2.3 Results and Discussion**

### *2.3.1 The Kinetically-Controlled Synthesis of Ampicillin*

Shown in Figure 2.3 are characteristic concentration profiles for the components of the kinetically-controlled synthesis of ampicillin from 6-APA and (R)-PGME utilizing iPGA. Figure 2.3 shows that ampicillin is initially produced via the synthesis reaction, achieves a maximum yield and then is completely consumed by secondary hydrolysis. 6-APA is initially consumed by the synthesis reaction, its minimum concentration is achieved when ampicillin reaches its maximum yield, and then it is completely re-generated by secondary hydrolysis. (R)-phenylglycine ((R)-PG) is constantly produced by primary and secondary hydrolysis. Finally, (R)-PGME is constantly being consumed by primary hydrolysis or by the synthesis reaction.

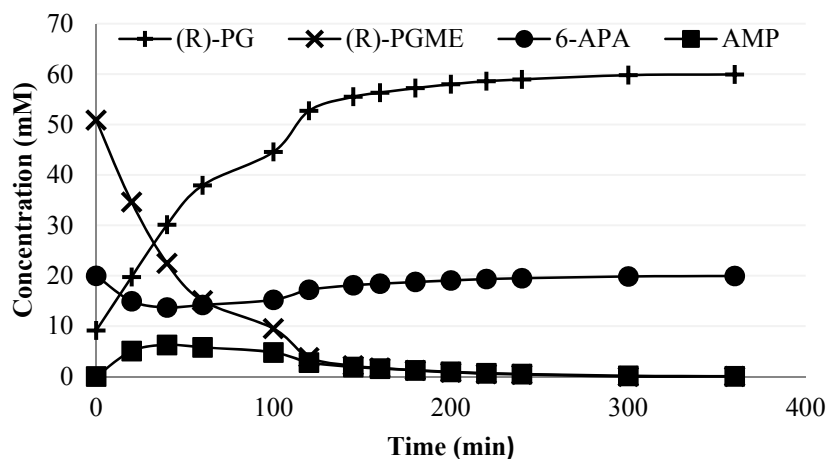


Figure 2.3: Characteristic concentration profiles observed in the kinetically-controlled synthesis of ampicillin (AMP) from 20 mM 6-APA and 60 mM (R)-PGME.

### 2.3.2 One-Pot Synthesis of Ampicillin

We evaluated both the 1P1S and 1P2S systems over a range of iPGA and AEH concentrations as shown in Table 2.1 and Table 2.2. In this cascade, enzyme concentrations have a large effect on the overall yield achieved and the degree of secondary hydrolysis observed. Typical reaction profiles for both configurations are shown in Figure 2.4.

It has been previously shown that the initial ratio of 6-APA to (R)-PGME is an important parameter in optimizing the coupling reaction for semi-synthetic antibiotics [17]. In our experiments we used 20 mM 6-APA and 60 mM (R)-PGME which has been demonstrated as the optimal ratio for both iPGA and AEH-catalyzed syntheses [15].

#### 2.3.2.1 One-Pot, One-Step Synthesis

The two-enzyme 1P1S system resulted in ampicillin yields between 6% and 39%, as shown in Table 2.1 and Figure 2.5A. The system performed poorly with low iPGA

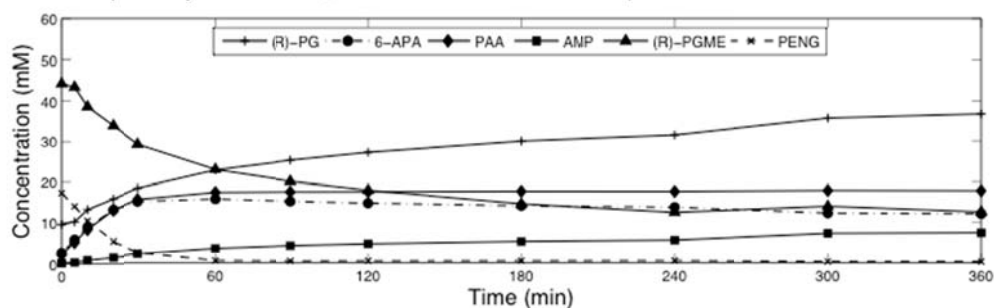
enzyme loading (22 UPenG) and high AEH enzyme loading (11 UAmp). AEHs have excellent (R)-PGME hydrolytic activity ( $k_{\text{cat}} = 982 \text{ s}^{-1}$ ) [15], thus the majority of the (R)-PGME was hydrolyzed prior to the production 6-APA that is necessary for synthesis. Increased iPGA enzyme loading (99 UPenG) and decreased AEH enzyme loading (between 1.1 UAmp – 5.5 UAmp) improved the ampicillin yields, with an optimum of 39% yield when 99 UPenG iPGA and 4.4 UAmp AEH was utilized. In the one-enzyme 1P1S system with iPGA the reactions only achieved a maximum conversion of 10% after 24 hours. The reduced reaction yield using iPGA alone was expected, due to the strong inhibition of iPGA with the intermediate PAA and the preference of iPGA for penicillin G ( $K_M=0.013\text{mM}$ ) over (R)-PGME ( $K_M=12.5 \text{ mM}$ ).

Table 2.1: Conversion results from the one-pot, one-step (1P1S) reaction configuration.

Enzyme Loading <sup>[a]</sup>		Time <sup>[b]</sup> (min)	Moles of (R)-PGME per mole of AMP at max. (mol /mol)	Maximum Conversion <sup>[c]</sup> (%)
iPGA (UPenG)	AEH (UAmp)			
24.8	11	20	48	6
99.2	1.1	360	8.7	23
99.2	2.2	300	6.3	38
99.2	4.4	60	7.5	39
99.2	5.5	60	11	30
99.2	none	360	31	3
114	none	1500	21	10
129	none	1500	20	9
136	none	360	25	5

[a] In ampicillin synthesis reactions starting from 6-APA and (R)-PGME, 1 UAmp of AEH  $\approx$  6.8 UPenG of iPGA; [b] Time reported is the time that the maximum conversion was observed; [c] Conversions are based on the moles of ampicillin produced per moles of penicillin G starting material. All concentrations are based on analytical measurements not isolated yields.

A. 1P1S Ampicillin Synthesis Profile, 99.2 UPenG iPGA and 2.2 UAmp AEH



B. 1P2S Ampicillin Synthesis Profile, 99.2 UPenG iPGA and 2.2 UAmp AEH

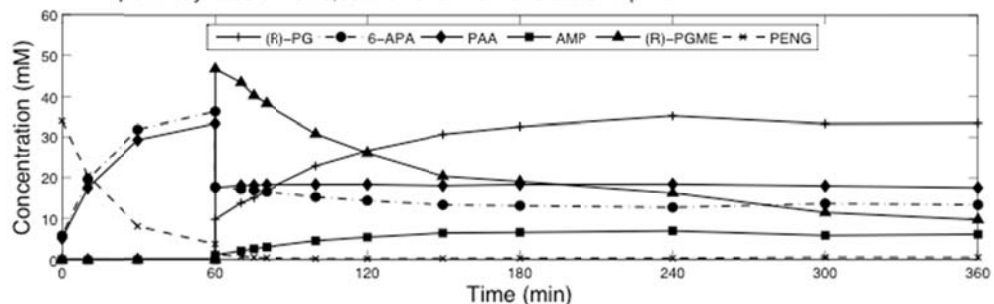


Figure 2.4: Reaction profile the enzymatic conversion of penicillin using 99.2 UPenG of iPGA and 2.2 of UAmp AEH. Both the 1P1S (A) and the 1P2S (B) profiles are shown. The components are (R)-PG, 6-APA, PAA, ampicillin (AMP), (R)-PGME and penicillin G (PENG).

### 2.3.2.2 One-Pot, Two-Step Synthesis

The two-enzyme 1P2S system resulted in ampicillin yields between 27% and 47% as shown in Table 2.2 and Figure 2.5B. Several configurations of enzyme loadings led to yields around 47%, which is equivalent to the observed yields when catalyzing the synthesis reaction with AEH directly from 6-APA and (R)-PGME [15]. In the 1P2S system the enzyme loading of AEH mostly impacted the secondary hydrolysis and decreased AEH loadings (between 1.1 and 4.4 UAmp) reduced the amount of secondary hydrolysis observed. The optimal configuration resulted in a 47% yield with minimal secondary hydrolysis and was observed when 99 UPenG iPGA and 4.4 UAmp AEH was utilized. Similarly to the 1P1S configuration, the single enzyme systems using iPGA resulted in low yield with a maximum conversion of 15% after 23 hours.

Table 2.2: Conversion results from the one-pot, two-step (1P2S) reaction configuration.

Step 1 Enzyme Loading <sup>[a]</sup>	Step 2 Enzyme Loading <sup>[a]</sup>		Step 1	Step 2 <sup>[b]</sup>	Total <sup>[b]</sup>	Moles of (R)-PGME per mole of AMP at max. (mol /mol )	Maximum Conversion <sup>[c]</sup> (%)
iPGA (UPenG)	iPGA (UPenG)	AEH (UAmp)	Time (min)	Time (min)	Time (min)		
24.8	none	11	145	15	160	6.0	47
99.2	none	1.1	60	300	360	6.9	27
99.2	none	2.2	60	180	240	6.3	35
99.2	none	4.4	60	90	150	6.2	46
99.2	none	5.5	60	30	90	6.1	47
24.8 <sup>[d]</sup>	none	11	130	20	150	6.1	45
24.8	74	none	130	410	540	15	6
99.2	15	none	60	1290	1350	17	12
99.2	30	none	60	1290	1350	15	14

[a] In ampicillin synthesis reactions starting from 6-APA and (R)-PGME, 1 UAmp of AEH  $\approx$  6.8 UPenG of iPGA; [b] Time reported is the time that the maximum conversion was observed; [c] Conversions are based on the moles of ampicillin produced per moles of penicillin G starting material. All concentrations are based on analytical measurements not isolated yields; [d] iPGA removed from the second step using filtration in the one-pot, two-step, two-stage process

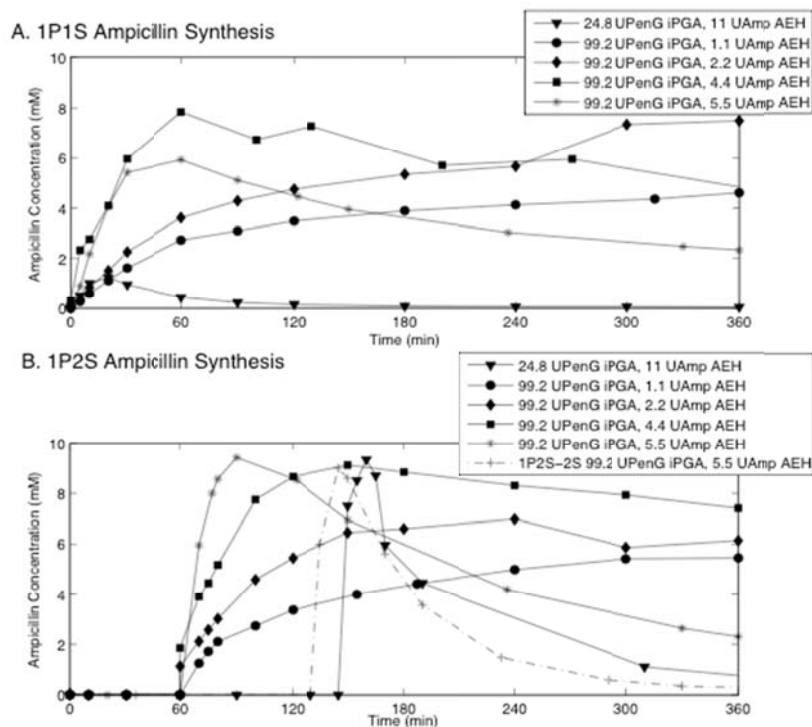


Figure 2.5: Ampicillin conversion profiles for both the 1P1S and 1P2S configurations. In the 1P2S reaction profiles there is no observed ampicillin until the second reaction step that was initiated 60 to 140 minutes into the reaction.



To investigate the impact of the excess iPGA on the secondary hydrolysis in the system we conducted a one-pot, two-step, two-stage scheme where iPGA was removed by filtration prior to the addition of AEH to the system in the second step. The removal of iPGA did not reduce the observed secondary hydrolysis of ampicillin, and therefore was not deemed beneficial to the 1P2S scheme.

The 1P1S system required fewer manipulations and has an overall faster cycle time but resulted in a lower overall yield when compared to the 1P2S system. The lower observed yields are likely due to the lower initial 6-APA nucleophile concentrations as 6-APA is generated at the same time it is consumed. The 1P2S step system required higher cycle times but results in higher overall yields and allowed for the most control of the system parameters, including the 6-APA to (R)-PGME ratio, when compared to the 1P1S system.

## **2.4 Conclusions**

The 1P1S scheme and the 1P2S scheme resulted in optimum ampicillin yields of 39% and 47%, respectively. In all cases, the two-enzyme system with iPGA and AEH out-performed the systems that used only iPGA, thus demonstrating the clear advantage of using AEH. While the 1P1S system resulted in slightly lower yields, it could be advantageous due to its operational ease and faster cycle times. In the 1P2S system, higher conversion was achieved and secondary hydrolysis was minimized by adjusting the relative enzyme loadings. These reaction schemes could be easily scaled up and incorporated with enzyme reuse, which has been previously demonstrated for iPGA[12, 18].

## **2.5 Publication Information**

The discussion of thermodynamic and kinetic syntheses of  $\beta$ -lactam antibiotics is published in Wiley's Encyclopedia of Industrial Biotechnology (Ed. M.C. Flickenger, 2010, pp 535-567) with Janna K. Blum and Andreas S. Bommarius as co-authors. The research presented in Chapter 2 of this dissertation on the one-pot synthesis of ampicillin using AEH and PGA was published in ChemCatChem (Vol. 2, Issue 8, pp 987-991) with Janna K. Blum, Carolina V. Perez, and Andreas S. Bommarius as co-authors. The date of online publication was July 28, 2010.

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## CHAPTER 3

### IMPROVING THE ENZYMATIC SYNTHESIS OF $\beta$ -LACTAM ANTIBIOTICS VIA MEDIUM ENGINEERING

#### 3.1 Introduction

There are many advantages for enzymatic syntheses in organic solvents including increased solubilities of reactants and products, shifting reaction equilibria, and most importantly for the enzymatic coupling step in  $\beta$ -lactam antibiotic synthesis, suppressing hydrolysis reactions. However, most enzymes show significantly decreased activity in organic co-solvents [1]. There have been numerous studies on the effects of different organic co-solvents on the synthesis of semi-synthetic  $\beta$ -lactam antibiotics [2-13]. Pan et al. studied the effect of the synthesis of ampicillin in fully organic media and attempted to correlate their results for yield with log P values. They were able to obtain a yield of 37.6% in ethyl acetate with a 24 hour reaction time and were not able to correlate their results with log P values [12]. van Langen et al. examined the synthesis of ampicillin in systems with less than 15% (v/v) water and found the more stable penicillin G acylase (PGA) from *Alcaligenes faecalis* to achieve 86% yield in 92% acetonitrile, though the beginning substrate concentrations were very low, 1 mM 6-aminopenicillanic acid and 3 mM (*R*)-phenylglycine amide [7]. More commonly, the enzymatic synthesis of  $\beta$ -lactam antibiotics is studied in partially organic media. Water miscible co-solvents, and more specifically polyols, are cited as suitable media for these syntheses [3, 4]. Ethylene glycol, methanol, 1,2-propanediol, 1,3-butanediol and glycerol, have all been reported to be advantageous for improving the synthesis of  $\beta$ -lactam antibiotics in terms of suppressing primary and secondary hydrolysis or improving yield [4, 6, 8, 9, 13]. Of

these co-solvents, ethylene glycol has been most studied because it has been proven to be the most effective at improving the yield while maintaining productivity for  $\beta$ -lactam antibiotic syntheses.

Given the current state of the literature, we began our investigation into medium engineering by examining the effects of ethylene glycol on the kinetically-controlled synthesis of ampicillin from (*R*)-phenylglycine methyl ester ((*R*)-PGME) and 6-aminopenicillanic acid (6-APA) catalyzed by penicillin G acylase (PGA). Our initial studies revealed that an extra product is formed in synthesis reactions where ethylene glycol was employed as a solvent. To our knowledge, this product has never been reported or studied. The present work outlines how we identified the extra product as (*R*)-phenylglycine hydroxyethyl ester ((*R*)-PGHEE), why we hypothesized that the formation of (*R*)-PGHEE could be responsible for the yield enhancement observed in the synthesis of ampicillin from (*R*)-PGME and 6-APA with ethylene glycol as a co-solvent, how we characterized the PGA-mediated hydrolysis and ampicillin synthesis potential of (*R*)-PGHEE, and how we sought to find systems to act as positive and negative controls to test our hypothesis.

## **3.2 Materials and Methods**

### *3.2.1 Materials*

6-aminopenicillanic acid (6-APA), (*R*)-phenylglycine ((*R*)-PG), ampicillin, (*R*)-phenylglycine methyl ester hydrochloride ((*R*)-PGME), (*R*)-phenylglycine amide ((*R*)-PGA), ethylene glycol, ethanol, tert-butyl alcohol, 2-nitro-5-[(phenylacetyl)amino]-

benzoic acid (NIPAB), and Amberlyst® 15 were procured from Sigma Aldrich (St. Louis, MO, USA).

### 3.2.2 Strains and Plasmids

The *pac* gene encoding penicillin G acylase has two mutations resulting in a LAla37Val substitution in the linker region and a  $\beta$ Val148Leu substitution in the mature protein as compared to the Swiss-Prot entry P06875 derived from *E. coli* ATC11105.

### 3.2.3 Expression of Penicillin G Acylase

PGA was expressed in 500 mL cultures. An overnight culture containing 5 mL LB media with 35  $\mu\text{g mL}^{-1}$  chloramphenicol was inoculated from a single HB101 *E. coli* colony and grown overnight at 37°C with agitation at 300 RPM. A 500 mL LB culture containing 35  $\mu\text{g mL}^{-1}$  chloramphenicol was seeded from the overnight culture and grown at 37°C with agitation at 300 RPM to an OD<sub>600</sub> of at least 0.8. Subsequently, the expression culture was induced with 0.1 mM IPTG and incubated at 17°C and with agitation at 250 RPM for 18 hours. After 18 hours, osmotic shock was performed by centrifugation at 3000 RPM (2280 x g) to obtain cell pellets; re-suspension of cell pellets in 5X volume of chilled osmotic shock buffer composed of 20% (w/v) sucrose, 100 mM TrisHCl and 10 mM EDTA at pH 8.0; centrifugation at 3000 RPM (2280 x g) to obtain cell pellets; re-suspension in 5X volume of chilled 1 mM EDTA to release the periplasmic fraction; and centrifugation at 3000 RPM (2280 x g) to pellet cell debris [14].

### 3.2.4 Ampicillin Synthesis Reactions

Periplasmic extract containing PGA was used for reactions containing varying concentrations of (R)-PGME and 6-APA per Tables 3.1; and varying concentrations of (R)-PGA and 6-APA per Table 3.2. Reactions were performed in 100 mM phosphate buffer with varying volume percentages of ethylene glycol, ethanol, or tert-butyl alcohol. All reactions were at 25°C and pH 7.0, contained 37 µg of active PGA variant, and had a total volume of 1600 µL. Reaction samples were taken at various points during the reaction and were quenched by diluting 10-fold with the addition of high performance liquid chromatography (HPLC) eluent for analysis with HPLC.

### 3.2.5 High Performance Liquid Chromatography

HPLC analysis was completed with a Shimadzu-LC-20AT pump, Beckman Coulter Ultrasphere ODS 4.6 mm x 25 cm column, and SPD-M20A prominence diode array detector monitored at 215 nm. The mobile phase was isocratic at 1.0 mL min<sup>-1</sup> with 30% acetonitrile and 70% 5 mM phosphate buffer containing 300 mg L<sup>-1</sup> sodium dodecyl sulfate at pH 2.65. (R)-PG, 6-APA, ampicillin, (R)-PGA, (R)-PGHEE, and (R)-PGME were analyzed on the HPLC. See Appendix B for more information on analytical methods [15].

### 3.2.6 Synthesis of (R)-Phenylglycine Hydroxyethyl Ester

(R)-Phenylglycine hydroxyethyl ester ((R)-PGHEE) was synthesized by reacting 2 g of (R)-PGME with 15 mL of ethylene glycol at 120°C with the aid of 500 mg of Amberlyst® 15 catalyst until the reaction was near completion as assayed by HPLC. The



product mixture was diluted with water to a volume of 50 mL, and extracted with 100 mL of 1 M potassium carbonate, followed by three extractions with 50 mL of dichloromethane. Dichloromethane was removed by vacuum. The product was identified by nuclear magnetic resonance,  $C_{10}H_{13}NO_3$ ,  $^1H$  NMR (acetone- $d_6$ ):  $\delta = 3.55$  (4H),  $\delta = 4.10$  (2H),  $\delta = 4.60$  (1H),  $\delta = 7.34$  (5H). The resulting product was greater than 90% pure as assayed by HPLC. Hydrolysis reactions and ampicillin synthesis reactions with (R)-PGHEE were carried out at 25°C and pH 7.

### *3.2.7 Chromogenic Activity Assay with 2-Nitro-5-[(Phenylacetyl)amino]-Benzoic Acid*

2-Nitro-5-[(phenylacetyl)amino]-benzoic acid (NIPAB), a chromogenic substrate and a penicillin G analog, was used to determine the stability of PGA in various organic solvents. PGA was incubated with different concentrations of organic co-solvents overnight and their residual activity on NIPAB was measured. The hydrolysis of NIPAB liberates 5-amino-2-nitrobenzoic acid, which was monitored at 405 nm with a Beckmann-Coulter DU®800 spectrophotometer. NIPAB hydrolysis reactions were carried out at 25°C in 50 mM phosphate buffer at pH 7.5 [16]. Detailed information for the NIPAB assay, along with other analytical techniques can be found in Appendix B.

### 3.3 Results and Discussion

#### 3.3.1 Synthesis of Ampicillin from (R)-Phenylglycine Methyl Ester and 6-Aminopenicillanic Acid with Ethylene Glycol Co-Solvent

##### 3.3.1.1 Synthesis of Ampicillin from (R)-Phenylglycine Methyl Ester and 6-Aminopenicillanic Acid with Varying Amounts of Ethylene Glycol Co-Solvent

The synthesis of ampicillin from 20 mM 6-APA and 60 mM (R)-PGME was investigated in amounts of ethylene glycol ranging from 0% (v/v) to 40% (v/v) as shown in Figure 3.1. As expected, an increase in yield, a decrease the rates of primary and secondary hydrolysis, and a decrease in the rate of ampicillin synthesis were observed with increasing amounts of ethylene glycol. The stability of PGA does not affect the rate of the synthesis of ampicillin, as PGA retains 100% of its activity on NIPAB after incubation with up to 50% (v/v) ethylene glycol overnight. 30% (v/v) ethylene glycol was chosen for further study as it still maintained an acceptable rate of synthesis while yielding an elevated amount of ampicillin.

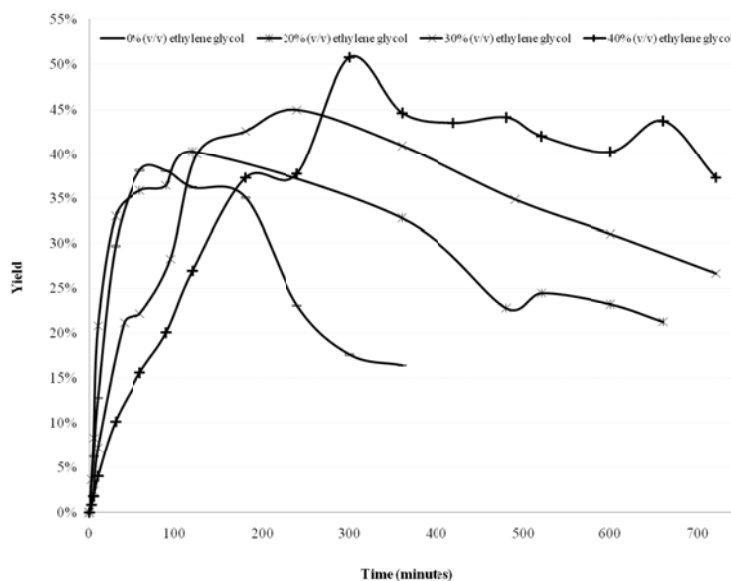


Figure 3.1: Ampicillin synthesis from 20 mM 6-APA and 60 mM (R)-PGME with varying amounts of ethylene glycol co-solvent.

### 3.3.1.2 Synthesis of Ampicillin from Varying Ratios of (R)-Phenylglycine Methyl Ester and 6-Aminopenicillanic Acid in Buffer and with Ethylene Glycol Co-Solvent

The synthesis of ampicillin in 0% (v/v) ethylene glycol and 30% (v/v) ethylene glycol was studied with nucleophile to electrophile ratios, or 6-APA to (R)-PGME ratios (6-APA:(R)-PGME), ranging from 0.33 to 3.0. As shown in Figure 3.2A and Table 3.1, the highest yield in phosphate buffer is 38% and is achieved with 20 mM 6-APA and 60 mM (R)-PGME, and the second best performing reaction achieves a yield of 34% with 60 mM 6-APA and 20 mM (R)-PGME. Interestingly, our results show that the yield achieved is highest for the lowest 6-APA:(R)-PGME when 6-APA:(R)-PGME is less than one, and is highest for the highest 6-APA:(R)-PGME when 6-APA:(R)-PGME is greater than one. Results from reactions with a 6-APA:(R)-PGME equal to one, namely those that had 60 mM of each reactant or 45 mM of each reactant, achieved yields of 26% and 29%, respectively, and eliminate the possibility that the yields are simply enhanced by increasing the amount of total substrate. These results suggest that two approaches can be taken to enhance the yields achieved in the kinetically-controlled synthesis of ampicillin in buffer. First, an excess of nucleophile can be used to ensure that when an acyl-enzyme intermediate forms, a  $\beta$ -lactam nucleophile will be present to deacylate the enzyme resulting in a synthesis reaction. The second approach, which is more effective, is to use an excess of the electrophile acyl side chain donor to compensate for the acyl donor that is lost to hydrolysis. The advantageous choice to use an excess of acyl donor is further supported by the facts that the hydrolysis product, (R)-phenylglycine ((R)-PG), can be recycled, and that 6-APA has limited stability and should be converted as much as possible in one pass [17].

As shown in Figure 3.2B and Table 3.1, the highest yield in 30% (v/v) ethylene glycol is 45% and is achieved with 20 mM 6-APA and 60 mM (R)-PGME, which equates to a 7 percentage point yield improvement as compared to the reaction performed in buffer with the same 6-APA:(R)-PGME. In contrast to the reactions in buffer, the second highest yielding reaction is achieved with 20 mM 6-APA and 45 mM (R)-PGME. A closer examination of the data shows that the difference in trends observed with 6-APA:(R)-PGME and yield in 0% (v/v) and 30% (v/v) ethylene glycol results from a variation in the benefit of utilizing ethylene glycol as a co-solvent with 6-APA:(R)-PGME as shown in Table 3.1. Specifically, the increase in percent yield observed for ampicillin synthesis performed with an ethylene glycol co-solvent was found to increase with the amount of (R)-PGME present in the reaction, while the increase in percent yield was found to be minimal, less than a three percentage points, for all reactions where 6-APA was in excess.

Table 3.1 Ampicillin synthesis with varying 6-APA:(R)-PGME in 0% (v/v) ethylene glycol, and in 30% (v/v) ethylene glycol

<u>6-APA:(R)-PGME</u>	<u>Concentration (mM)</u>		<u>Maximum Yield (%)</u>		<u>Δ Yield (% points)</u>
	6-APA	(R)-PGME	0% (v/v) Ethylene Glycol	30% (v/v) Ethylene Glycol	
0.33	20	60	38	45	7
0.44	20	45	30	35	5
0.61	20	33	23	27	4
1.00	20	20	17	18	1
1.65	33	20	22	24	2
2.25	45	20	27	29	2
3.00	60	20	34	34	1
1.00	45	45	26	29	3
1.00	60	60	29	32	3

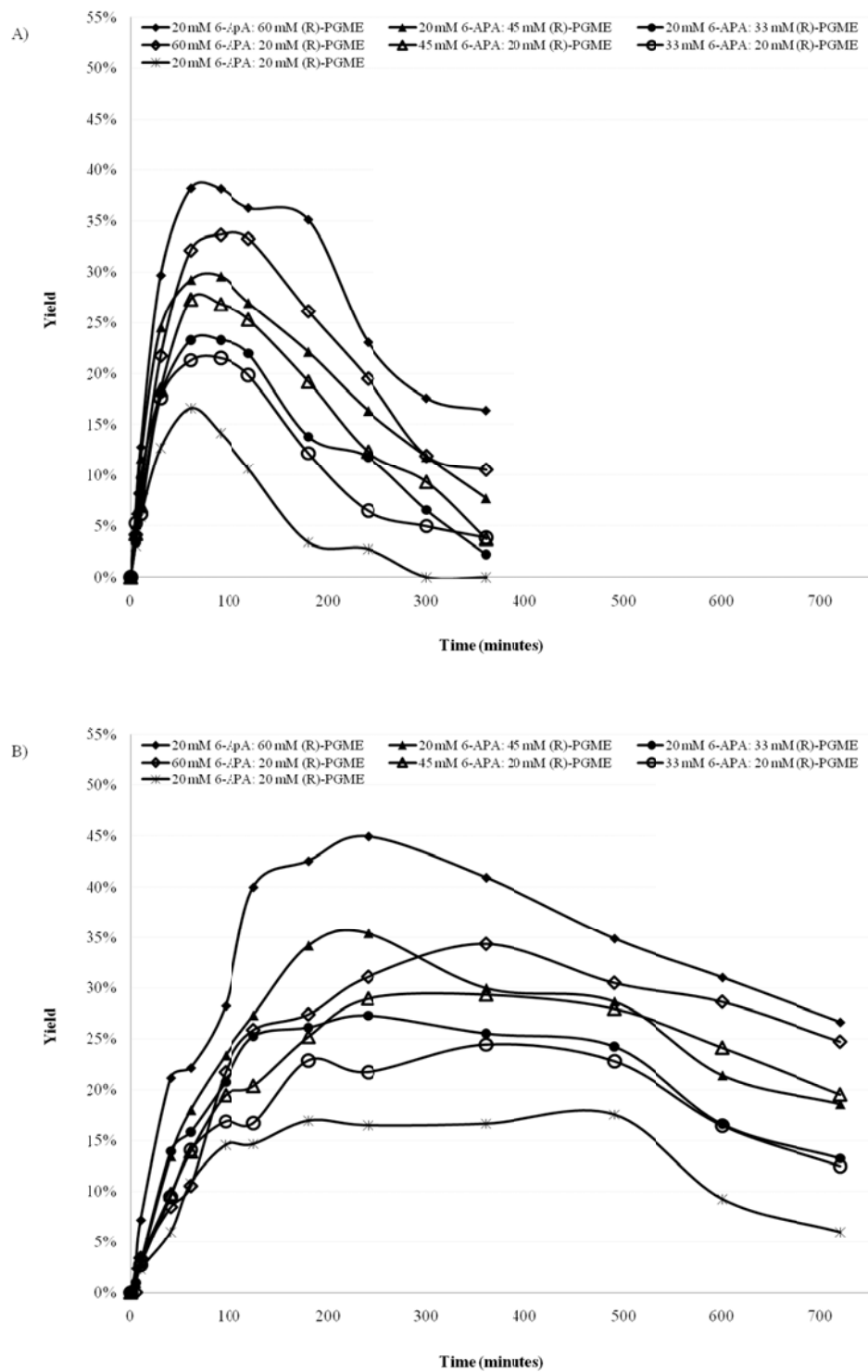


Figure 3.2 Ampicillin synthesis with varying 6-APA:(R)-PGME in A) 0% (v/v) ethylene glycol, and in B) 30% (v/v) ethylene glycol

### 3.3.1.3 Discovery of Intermediate Formed in Synthesis of Ampicillin from (R)-Phenylglycine Methyl Ester and 6-Aminopenicillanic Acid with Ethylene Glycol Co-Solvent

HPLC analysis showed an extra product forming in reactions where ethylene glycol was employed as a co-solvent. We hypothesized that the compound that is formed is the transesterification product of (R)-PGME and ethylene glycol, (R)-PGHEE, based on the following three observations: (1) the amount of product increased with the amount of (R)-PGME in the reaction, (2) the amount of product increased with the amount of ethylene glycol in the reaction, and (3) the product was present in systems containing only (R)-PGME and ethylene glycol but was not present in systems containing only 6-APA and ethylene glycol. (R)-PGME hydrolysis reactions in 0% (v/v) ethylene glycol and in 30% (v/v) ethylene glycol with and without the presence of PGA indicate that the formation of this product is not dependent on an enzymatic reaction, or that its enzymatically-catalyzed hydrolysis is much faster than the enzymatically-catalyzed synthesis. Interestingly, in the absence of PGA (R)-PGME is consumed at the same rate in 30% (v/v) ethylene glycol as it is in buffer.

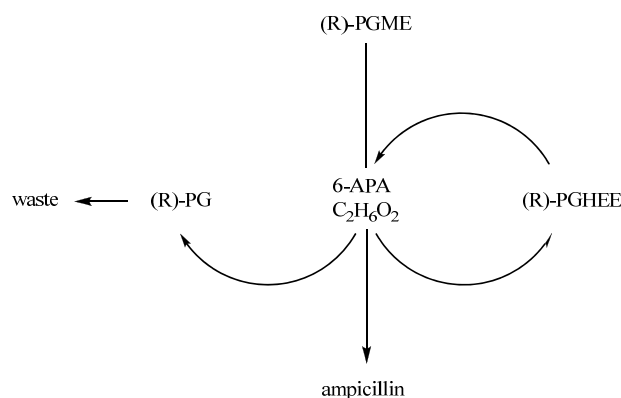


Figure 3.3 In-situ mixed donor synthesis of ampicillin with 6-APA and (R)-PGME with ethylene glycol ( $C_2H_6O_2$ ) co-solvent.

To our knowledge the formation of this product or its effect on the synthesis of ampicillin has never been reported. The concentration of the product is transient, indicating that it is formed and consumed during the course of the reactions. Since the specificity of PGA is limited to the phenylacetyl portion of its substrates, if the product formed in reactions containing ethylene glycol as a co-solvent is (R)-PGHEE, it could be employed by the enzyme to synthesize ampicillin. Thus, we hypothesized that the formation of (R)-PGHEE is contributing to the yield enhancement observed in the synthesis of ampicillin from (R)-PGME and 6-APA employing ethylene glycol as a co-solvent, based on the observations that: (1) the loss of acyl donor in the reaction affects the overall yield achieved, (2) the yield enhancement observed for the synthesis of ampicillin is dependent on the amount of (R)-PGME used for the synthesis reaction, (3) the most widely-reported type of co-solvent cited in the literature for ampicillin synthesis are polyols, which are all theoretically capable of transesterification of (R)-PGME, and (4) all instances reported in the literature for the improved synthesis of a  $\beta$ -lactam antibiotic as a result of the use of a polyol co-solvent have used an excess of (R)-PGME. Essentially, transesterification of (R)-PGME to (R)-PGHEE rather than hydrolysis of (R)-PGME to (R)-PG would re-direct the consumption of (R)-PGME to an intermediate that could be used to synthesize ampicillin, rather than to an unusable hydrolysis side product. The resulting system would represent an in-situ mixed donor synthesis, where both (R)-PGME and (R)-PGHEE act as side chain acyl donors to synthesize ampicillin as shown in Figure 3.3. A mixed donor synthesis from (*R*)-phenylglycine amide ((*R*)-PGA) and (R)-PGME has been reported previously as an ex-situ means to improve the rate of ampicillin

synthesis while maintaining equivalent yields and recycling the hydrolysis product (R)-PG [18].

### 3.3.2 Synthesis of Ampicillin from (R)-Phenylglycine Amide and 6-Aminopenicillanic Acid with Ethylene Glycol Co-Solvent

(R)-phenylglycine amide ((R)-PGA) is an alternative acyl side chain donor to (R)-PGME that can be used to synthesize ampicillin. The suppression of primary and secondary hydrolysis reactions is present in reactions with (R)-PGA when ethylene glycol is employed as a co-solvent, but (R)-PGA is more stable than (R)-PGME and we have found that it does not undergo transesterification with ethylene glycol. Therefore, the synthesis of ampicillin from (R)-PGA and 6-APA in 30% (v/v) ethylene glycol acts a negative control for the hypothesis that the formation of (R)-PGHEE is contributing to the yield enhancement observed in the synthesis of ampicillin from (R)-PGME and 6-APA.

Table 3.2 Ampicillin synthesis with varying 6-APA:(R)-PGA in 0% (v/v) ethylene glycol, and in 30% (v/v) ethylene glycol

6-APA:(R)-PGA	Concentration (mM)		Maximum Yield (%)		$\Delta$ Yield (% points)
	6-APA	(R)-PGA	0% (v/v) Ethylene Glycol	30% (v/v) Ethylene Glycol	
0.33	20	60	34	34	0
0.44	20	45	24	23	-1
0.61	20	33	17	19	2
1.00	20	20	10	13	3
1.65	33	20	18	19	1
2.25	45	20	20	23	3
3.00	60	20	27	30	3
1.00	45	45	21	23	2
1.00	60	60	25	26	1



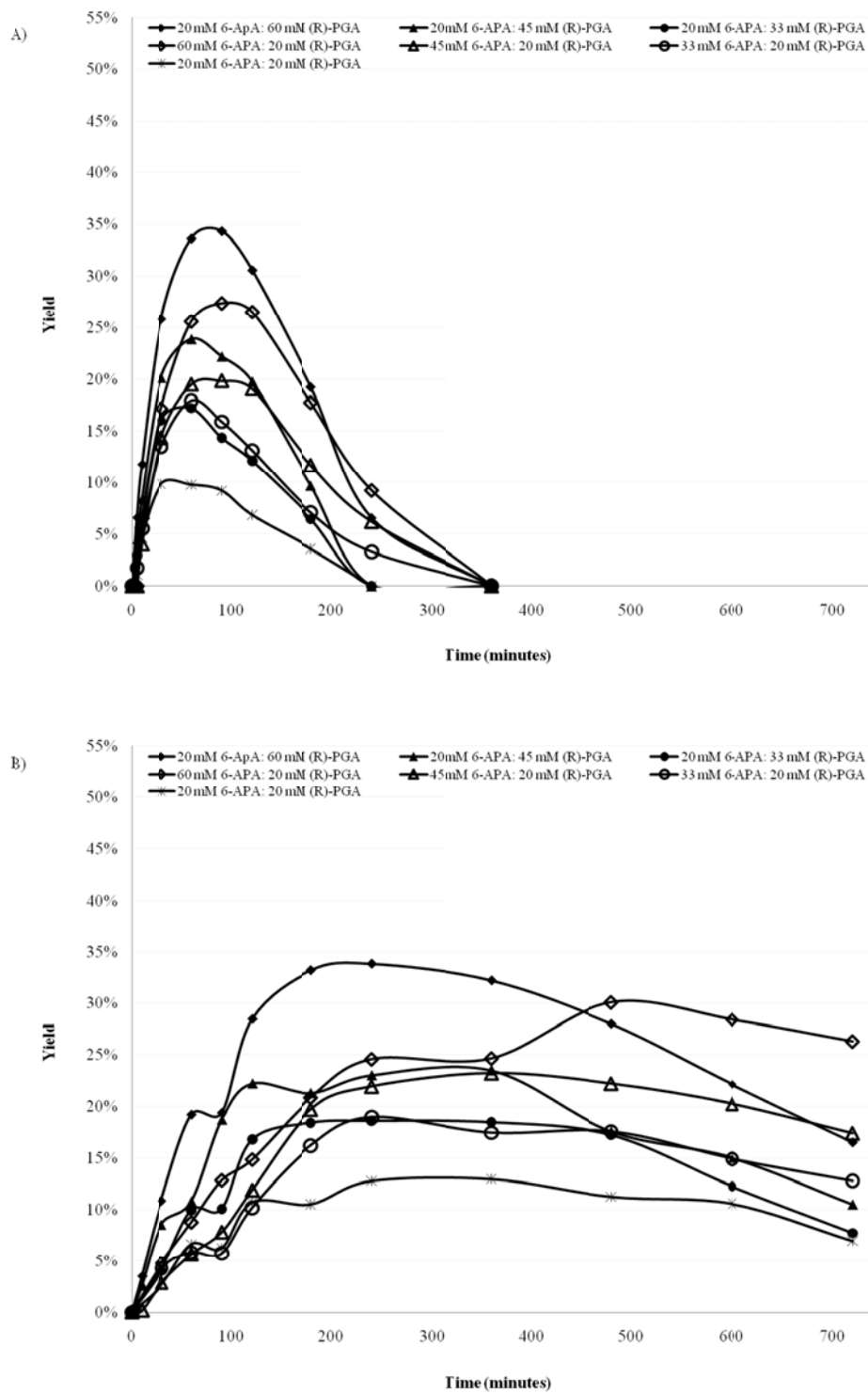


Figure 3.4 Ampicillin synthesis with varying 6-APA:(R)-PGME in A) 0% (v/v) ethylene glycol, and in B) 30% (v/v) ethylene glycol

To study the effects of the synthesis of ampicillin without the presence of (R)-PGHEE, the synthesis of ampicillin in 0% (v/v) ethylene glycol and 30% (v/v) ethylene glycol was studied with ratios of 6-APA to (R)-PGA (6-APA:(R)-PGA) ranging from 0.33 to 3.0. Figure 3.4A and Table 3.2 show that the trends in yield achieved with varying 6-APA:(R)-PGA in buffer are consistent with those observed for 6-APA:(R)-PGME. The best performing 6-APA:(R)-PGA was 20 mM 6-APA and 60 mM (R)-PGA which achieved a yield of 34%, followed by the reaction containing 60 mM 6-APA and 20 mM (R)-PGME which achieved a yield of 27%. Again, it was observed that the yield is highest the further the nucleophile to electrophile ratio is from unity. These results further support the finding that either an excess of nucleophile or an excess of electrophile should be used in the kinetically-controlled synthesis of ampicillin.

Interestingly, the trend observed with varying 6-APA:(R)-PGA in 30% (v/v) ethylene glycol is identical to that observed for 6-APA:(R)-PGA in buffer and 6-APA:(R)-PGME in buffer as shown in Figure 3.4B and Table 3.2. Furthermore, it was determined that the effect of using an ethylene glycol co-solvent only minimally increased the yield achieved, less than three percentage points, for all 6-APA:(R)-PGA studied.

### *3.3.3 Comparison of Ampicillin Synthesis from Different Activated Acyl Donors in Buffer and with Ethylene Glycol Co-Solvent*

To compare the syntheses with different activated acyl donors and the syntheses with and without employing ethylene glycol as a co-solvent, we will compare the four reactions that had a nucleophile to electrophile ratio of 0.33 as they led to the highest yielding reaction in all four sets of synthesis reactions studied. The initial rates of (R)-PG

generation, ampicillin generation, and (R)-PGME or (R)-PGA consumption for these four reactions can be found in Table 3.3.

Utilizing (R)-PGME as an activated acyl side chain donor leads to faster initial rates of synthesis and higher yields of ampicillin in comparison to using (R)-PGA for all of the nucleophile to electrophile concentrations studied in this work. In buffer, the synthesis with 20 mM 6-APA and 60 mM electrophile results in a 1.2-fold increase in the initial rate of synthesis and a maximum yield that is four percentage points greater when (R)-PGME is employed as opposed to (R)-PGA. In 30% (v/v) ethylene glycol, the synthesis with 20 mM 6-APA and 60 mM electrophile results in a 1.2-fold increase in the initial rate of synthesis and a maximum yield 11 percentage points greater when (R)-PGME is used as opposed to (R)-PGA. When (R)-PGME is the activated acyl side donor of choice, the initial rates of (R)-PG generation, ampicillin synthesis, and (R)-PGME consumption in 30% (v/v) ethylene glycol are 20%, 30%, and 41%, respectively, of the rates observed in buffer. Therefore, the rate of primary hydrolysis is suppressed to a greater extent than the rate of synthesis, which in combination with suppressed secondary hydrolysis, leads to the observed yield increase. When (R)-PGA is the activated acyl side donor of choice, the initial rates of (R)-PG generation, ampicillin synthesis, and (R)-PGA consumption in 30% (v/v) ethylene glycol are 28%, 27%, and 31%, respectively, of the rates observed in buffer. Therefore, in the reactions with (R)-PGA all reactions are impeded by the presence of ethylene glycol to an equal extent and therefore an enhancement in yield is not observed.

The PGA-catalyzed hydrolysis of 20 mM ampicillin was carried out to study the effect of ethylene glycol on the secondary hydrolysis reaction. The rate of ampicillin hydrolysis in 30% (v/v) ethylene glycol was found to be 26% of the rate in buffer, explaining the significant decrease in secondary hydrolysis for all reactions where ethylene glycol was employed as a co-solvent as shown in Figures 3.2 and 3.4.

Table 3.3 Synthesis of ampicillin from 20 mM 6-APA and 60 mM side chain acyl donor in 0% (v/v) ethylene glycol and 30% (v/v) ethylene glycol.

Medium	(R)-PG Generation (mM min <sup>-1</sup> )	Ampicillin Generation (mM min <sup>-1</sup> )	Acyl Donor Consumption (mM min <sup>-1</sup> )
<i>Ampicillin Synthesis from (R)-PGME</i>			
0% (v/v) ethylene glycol	0.325	0.256	0.616
30% (v/v) ethylene glycol	0.070	0.078	0.257
<i>Ampicillin Synthesis from (R)-PGA</i>			
0% (v/v) ethylene glycol	0.269	0.236	0.469
30% (v/v) ethylene glycol	0.076	0.064	0.143

### 3.3.4 Synthesis of Ampicillin from (R)-Phenylglycine Methyl Ester and 6-Aminopenicillanic Acid with Ethanol and tert-Butyl Alcohol Co-Solvents

To probe the extent to which transesterification of (R)-PGME by a co-solvent positively affects yield, the synthesis of ampicillin in other co-solvents bearing an alcohol group was investigated. Finding such a co-solvent would serve as a positive control to the reactions with an ethylene glycol co-solvent where (R)-PGHEE is present and the yield is positively affected. We limited our studies to ethanol and tert-butyl alcohol because their transesterification products with (R)-PGME are readily available.

#### 3.3.4.1 Synthesis of Ampicillin from (R)-Phenylglycine Methyl Ester and 6-Aminopenicillanic Acid with Ethanol Co-Solvent

The synthesis of ampicillin from 20 mM 6-APA and 60 mM (R)-PGME was investigated in amounts of ethanol ranging from 0% (v/v) to 40% (v/v). PGA was shown to retain 93% and 60% of its activity on NIPAB after overnight incubation with 20% (v/v) and 40% (v/v) ethanol, respectively. We found that the transesterification product of ethanol and (R)-PGME, (R)-phenylglycine ethyl ester ((R)-PGEE), was produced in reactions in 20% (v/v) and 40% (v/v) ethanol. However, the decrease in the rate of ampicillin synthesis in ethanol is drastic, only 6% of the initial synthetic activity is observed in 20% (v/v) ethanol as compared to buffer. As a result, the maximum yield achieved in 20% (v/v) or 40% (v/v) ethanol was lower than that observed in buffer, only 28.5% and 12.9%, respectively, after 12 hours. The initial rate in 10% (v/v) ethanol was 33% of that observed in buffer, but the maximum yield achieved was only 38.9%, representing a less than 1% yield enhancement as compared to reaction in buffer. Furthermore, an insignificant amount of (R)-PGEE was produced in 10% (v/v) ethanol. Because the transesterification of (R)-PGME was only present at ethanol concentrations that severely impeded the rate of ampicillin synthesis and maximum yield achieved, we determined that utilizing the co-solvent ethanol would not serve to prove or disprove that the transesterification by a co-solvent positively affect yield.

#### 3.3.4.2 Synthesis of Ampicillin from (R)-Phenylglycine Methyl Ester and 6-Aminopenicillanic Acid with tert-Butyl Alcohol Co-Solvent

The synthesis of ampicillin from 20 mM 6-APA and 60 mM (R)-PGME was investigated in amounts of tert-butyl alcohol ranging from 0% (v/v) to 30% (v/v).

However, tert-butyl alcohol was also found to severely impede PGA's synthetic activity for ampicillin synthesis. The rate of ampicillin synthesis was found to be only 21% and 11% of the rate observed in buffer for 10% (v/v) and 20% (v/v) tert-butyl alcohol, respectively, and the resulting yields were only 22% and 11%, respectively. Furthermore, we were not able to identify the formation of (R)-phenylglycine tert-butyl ester, the transesterification product between (R)-PGME and tert-butyl alcohol. These results indicate that tert-butyl alcohol also cannot serve as a co-solvent to prove or disprove that the transesterification of (R)-PGME by a co-solvent can positively affect yield in the synthesis of ampicillin.

### *3.3.5 Penicillin G Acylase's Activity for (R)-Phenylglycine Hydroxyethyl Ester*

#### 3.3.5.1 Hydrolysis of (R)-Phenylglycine Hydroxyethyl Ester

The chemical and enzymatic hydrolysis of 60 mM (R)-PGHEE was investigated. We found the PGA-catalyzed rate of (R)-PGHEE hydrolysis to be  $0.085 \text{ mM min}^{-1}$ , which is 20% and 27% of the rate of the enzymatic hydrolysis of (R)-PGME and (R)-PGA, respectively. The rate of chemical hydrolysis rate of (R)-PGHEE was determined to be  $0.056 \text{ mM min}^{-1}$ , surprisingly high at 66% of the total hydrolysis rate of (R)-PGHEE in the presence of PGA. For comparison, the rate of background hydrolysis of (R)-PGME is 13% of the rate of combined chemical and enzymatic hydrolysis, and the chemical hydrolysis of (R)-PGA does not occur at all under the reaction conditions and time scales that we employ.

### 3.3.5.2 Synthesis of Ampicillin from (R)-Phenylglycine Hydroxyethyl Ester and 6-Aminopenicillanic Acid

(R)-PGHEE was tested for its ability to synthesize ampicillin with 6-APA. (R)-PGHEE was found to be a viable acyl side chain donor for ampicillin synthesis. However, the rate of synthesis from 20 mM 6-APA and 60 mM (R)-PGHEE was only  $0.013 \text{ mM min}^{-1}$ , which is about 5% of the synthesis rates observed for (R)-PGME and (R)-PGA. This result is astounding given the fact that Bristol-Myers Squibb (Princeton, NJ, USA) has previously reported that ethylene glycol esters are its most preferred form of activated acyl side chain donors [19].

## **3.4 Conclusions**

In this work, we have investigated the kinetically-controlled synthesis of ampicillin with different activated acyl side chain donors in partially organic media. We have found that utilizing ethylene glycol as a co-solvent decreases the rates of primary and secondary hydrolysis observed in the kinetically-controlled synthesis of ampicillin. We have found that the primary hydrolysis observed in the synthesis from (R)-PGME is suppressed to a lesser extent than the primary hydrolysis observed in the synthesis from (R)-PGA. The suppression of secondary hydrolysis of ampicillin in the presence of ethylene glycol is equivalent for both reactions.

Furthermore, we have investigated the effect that transesterification by a co-solvent has on the yield enhancement observed in the synthesis of ampicillin. We have confirmed that ethylene glycol transesterifies (R)-PGME to intermittently produce (R)-PGHEE but does not transesterify (R)-PGA. Therefore, ampicillin synthesis with (R)-PGA in the presence of 30% (v/v) ethylene glycol represent a system that has suppressed

hydrolysis reactions in the absence of transesterification by a co-solvent. When (R)-PGA is used at the activated acyl side chain, we have found that the use of ethylene glycol as a co-solvent has minimal effects on the yield of ampicillin achieved, in all cases less than three percentage points. When (R)-PGME is used as the activated acyl side chain, we have found that yield enhancement observed when employing ethylene glycol as a co-solvent is dependent on the amount of (R)-PGME present.

Also, we have demonstrated that (R)-PGHEE is capable of acting as a side chain acyl donor to synthesize ampicillin. Based on our results, there is evidence that the synthesis of ampicillin from (R)-PGME and 6-APA with an ethylene glycol co-solvent acts as an in situ mixed donor synthesis, where both (R)-PGME and (R)-PGHEE are acyl side chain donors for the synthesis of ampicillin, and that the mixed donor synthesis contributes to the enhanced yield observed.

### **3.5 Publication Information**

The research presented in Chapter 3 of this dissertation will be submitted to Chemical Communications by August 10<sup>th</sup>, 2011 for publication with Andreas S. Bommarius as a co-author.



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## CHAPTER 4

### IMPROVING THE ENZYMATIC SYNTHESIS OF $\beta$ -LACTAM ANTIBIOTICS VIA DATA-DRIVEN PROTEIN ENGINEERING

#### 4.1 Introduction

Stereoselective processes for preparation of pharmaceuticals is especially important as stereoisomers often have different pharmacokinetic, pharmacologic, and toxicologic effects [1]. However, penicillin G acylase (PGA) exhibits relaxed diastereoselectivity and therefore enantiomerically pure acyl donor substrates must be prepared for the enzymatic synthesis of semi-synthetic  $\beta$ -lactam antibiotics. In this study, we aimed to use protein engineering to improve the selectivity of PGA with respect to the amino group on the alpha carbon ( $C_\alpha$ ) in the synthesis of ampicillin from *rac*-phenylglycine methyl ester (*rac*-PGME) and 6-aminopenicillanic acid (6-APA) so that racemic acyl donors can be used in the synthesis of  $\beta$ -lactam antibiotics.

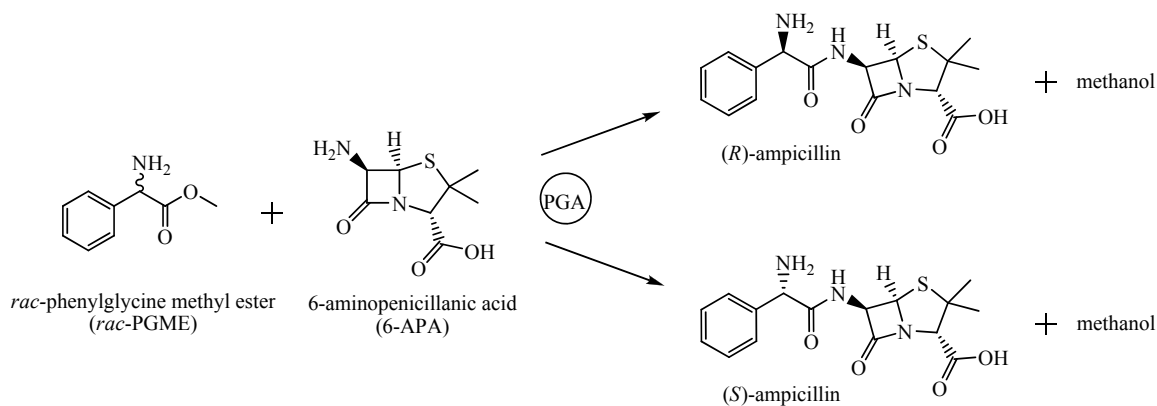


Figure 4.1: The synthesis of ampicillin from *rac*-PGME. Primary hydrolysis of *rac*-PGME and secondary hydrolysis of ampicillin are not shown for simplicity.

Currently, when starting with *rac*-PGME and 6-APA, PGA will catalyze the synthesis of the desired product, (*R*)-ampicillin, in addition to an undesired product, (*S*)-ampicillin, as shown in Figure 4.1. PGA shows an initial diastereomeric excess (d.e.) of 15% and a d.e. value of 37% at the point when the maximum yield of (*R*)-ampicillin is achieved, both with respect to the desired synthesis product, (*R*)-ampicillin. Morley and Kazlauskas found that mutations close to the active site are most effective in altering enzyme selectivity [2]. Therefore to alter the selectivity of PGA we targeted residues based their proximity to the C<sub>α</sub> of the inhibitor penicillin G sulfoxide for site-saturation with NNK libraries (N represents an equal mixture of the nucleotide bases A, G, C, and T; and K represents an equal mixture of T and G). Residues that are known to be necessary for catalysis were not considered for mutation. The five residues that are within 6 Å of the C<sub>α</sub> penicillin G sulfoxide and therefore were chosen to study for diastereomer selectivity improvement are αPhe146, βPro22, βPhe24, βThr68, and βPhe71 (the amino acid residues are labeled to indicate the chain, α or β, the wild type amino acid residue, the residue number on the chain, and the variant amino acid residue when applicable). Figure 4.2 shows the residues chosen for mutation in the active site of PGA. Though little research has been completed on the selectivity of PGA with respect to the substituent on the C<sub>α</sub>, extensive research on the first shell residues in the active site of PGA has been completed. Previous alignment of eight known class IIa PGA sequences shows that in comparison to the wild-type residues of PGA from *E. coli*, αPhe146 is 37.5% conserved, βPro22 is 100% conserved, βPhe24 is 75% conserved, βThr68 is 100% conserved, and βPhe71 is 62.5% conserved [3]. Residue αPhe146 is known to act as a ‘gatekeeper’ to the active site because of its ability to occupy two different energetically

favored positions: the residue is in a 'closed' helical conformation and shields the hydrophobic active site from solvent when either no substrate is present or a tightly bound small substrate is present, or the residue shifts out towards the solvent in an 'open' coil conformation when a weakly bound large substrate is present. When a  $\beta$ -lactam antibiotic is bound, the  $\alpha$ Phe146 side-chain interacts with the thiazolidine ring of the  $\beta$ -lactam nucleus [4-6].  $\alpha$ Phe146 and  $\beta$ Phe24 are on opposite sides of the substrate binding pocket as shown in Figure 4.2. Their aromatic rings are known to interact with the phenylacetyl side-chain of substrates and to limit the size of the  $C_{\alpha}$ -substituent that can bind in phenylacetic acid derivatives [6, 7].  $\beta$ Phe71 is known to undergo a change in conformation upon binding with large substrates and forms a stacked conformation with thiazolidine ring of the  $\beta$ -lactam nucleus. Additionally,  $\beta$ Phe71 and  $\alpha$ Phe146 are known to be structurally linked via a calcium ion [4, 5].  $\beta$ Pro22 is known to be part of the hydrophobic substrate binding pocket [7].

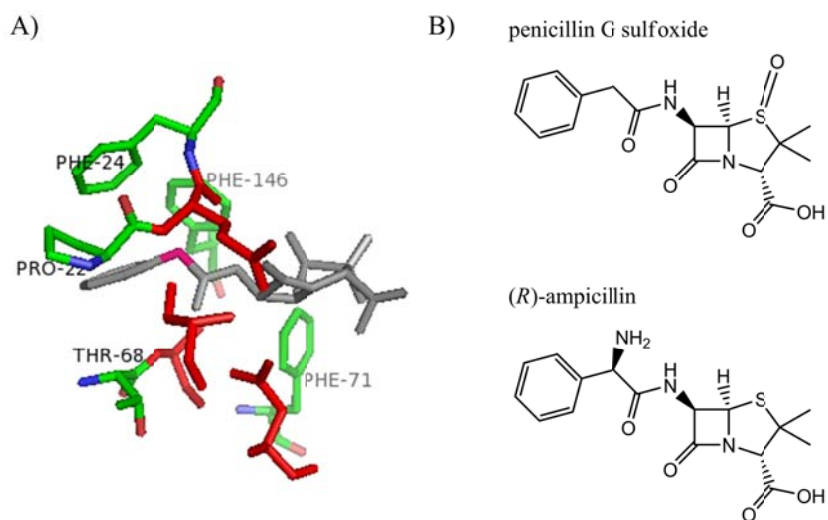


Figure 4.2: A) Crystal structure showing the active site of PGA complexed with penicillin G sulfoxide (PDB 1GM9). Penicillin G sulfoxide is shown in grey with the  $C_{\alpha}$  highlighted in pink. Residues that were chosen for mutation are labeled and residues that are known to be necessary for catalysis are shown in red. B) The structures of penicillin G sulfoxide and (R)-ampicillin

Only two studies on the selectivity of PGA with respect to substituents on C $_{\alpha}$  position have been completed. Švedas, et al. completed an extensive study on the substrate specificity of wild-type PGA and found that PGA did not have any significant stereospecificity for substrates with the polar groups –OH or –NH<sub>2</sub> at the C $_{\alpha}$  position [8]. Alkema et al. studied the selectivity of wild-type PGA and the PGA variants  $\beta$ Phe24Ala,  $\alpha$ Phe146Tyr, and the double variant  $\beta$ Phe24Ala/ $\alpha$ Phe146Tyr with various C $_{\alpha}$ -substituted substrates, though the selectivity with an amino group substituent was not studied. The selectivity of wild-type PGA was found to be dependent on the size and polarity of the substituent, PGA variant  $\beta$ Phe24Ala was found to increase enantioselectivity towards all (*R*)-enantiomers, variant  $\alpha$ Phe146Tyr was shown to have enantioselectivity similar to the wild-type, and double variant  $\beta$ Phe24Ala/ $\alpha$ Phe146Tyr was shown to have additive enantioselective properties of the two single point mutations [7]. Both studies investigated the enantioselectivity of PGA by utilizing enantiomeric substrates as inhibitors, experimentally determining their inhibition constants, and comparing them to determine the enzyme's enantioselectivity.

To our knowledge, the present work is the first study to investigate and improve the selectivity of PGA with respect to substrates' C $_{\alpha}$  with site-saturation of individual residues. Furthermore, this research represents the first time that the selectivity of PGA with respect to the C $_{\alpha}$  has been investigated for a synthesis reaction to produce a diastereomerically pure semi-synthetic  $\beta$ -lactam antibiotic.

## 4.2 Materials and Methods

### 4.2.1 Materials

6-aminopenicillanic acid (6-APA), (*R*)-phenylglycine ((*R*)-PG), ampicillin, (*R*)-phenylglycine methyl ester hydrochloride ((*R*)-PGME), (*S*)-phenylglycine methyl ester hydrochloride ((*S*)-PGME), phenylmethanesulfonyl fluoride (PMSF), and 2-nitro-5-[(phenylacetyl)amino]-benzoic acid (NIPAB) were procured from Sigma Aldrich (St. Louis, MO, USA).

### 4.2.2 Strains and Plasmids

The *pac* gene encoding penicillin acylase has two mutations resulting in a LAla37Val substitution in the linker region and a  $\beta$ Val148Leu substitution in the mature protein as compared to the Swiss-Prot entry P06875 derived from *E. coli* ATC11105.

### 4.2.3 Mutagenesis

Diverse libraries were generated by following the protocol of QuikChange<sup>TM</sup> Site-Directed Mutagenesis Kit. The forward and reverse primers utilized to create the  $\alpha$ Phe146 library were 5'-GC ACC ATG GCA AAC CGC NNK TCG GAT AGC ACT AGC GAA ATC G-3' and 5'-C GAT TTC GCT AGT GCT ATC CGA MNN GCG GTT TGC CAT GGT GC-3', respectively. The forward and reverse primers utilized to create the  $\beta$ Pro22 library were 5'-CG AAA GCA ATC ATG GTA AAT GGT NNK CAA TTT GGC TGG TAT GCG C-3' and 5'-G CGC ATA CCA GCC AAA TTG MNN ACC ATT TAC CAT GAT TGC TTT CG-3', respectively. The forward and reverse primers utilized to create the  $\beta$ Phe24 library were 5'-CA ATC ATG GTA AAT GGT CCG CAG

NNK GGT TGG TAT GCG CCT GCG-3' and 5'-CGC AGG CGC ATA CCA ACC MNN CTG CGG ACC ATT TAC CAT GAT TG-3', respectively. The forward and reverse primers utilized to create the  $\beta$ Thr68 library were 5'-GGT GTG ATT TCC TGG GGA TCA NNK GCT GGT TTC GGC GAT GAT G-3' and 5'-C ATC ATC GCC GAA ACC AGC MNN TGA TCC CCA GGA AAT CAC ACC-3', respectively. The forward and reverse primers utilized to create the  $\beta$ Phe71 library were 5'-C TGG GGA TCA ACG GCA GGT NNK GGT GAT GAT GTC GAT ATT TTT GCT G-3' and 5'-C AGC AAA AAT ATC GAC ATC ATC ACC MNN ACC TGC CGT TGA TCC CCA G-3', respectively. The point mutation  $\beta$ Phe24Ala was also generated by following the protocol of QuikChange<sup>TM</sup> Site-Directed Mutagenesis Kit. The forward and reverse primers utilized to create the  $\beta$ Phe24Ala were 5'-CA ATC ATG GTA AAT GGT CCG CAG GCG GGT TGG TAT GCG CCT GCG-3' and 5'-CGC AGG CGC ATA CCA ACC CGC CTG CGG ACC ATT TAC CAT GAT TG-3', respectively. The resulting PCR products were transformed into competent XL1-Blue *E. coli* cells and grown overnight in LB media containing 35  $\mu\text{g mL}^{-1}$  chloramphenicol at 37°C. Liquid cultures were subsequently mini-prepped, transformed into competent HB101 *E. coli* cells, and grown overnight on agar plates containing 35  $\mu\text{g mL}^{-1}$  chloramphenicol. Library diversity was confirmed with sequencing.

#### 4.2.4 Expression of Penicillin G Acylase and Penicillin G Acylase Variants

Libraries of PGA variants were expressed in 96-well microtiter plates. Master 96-well microtiter plates containing 170  $\mu\text{L}$  LB media with 35  $\mu\text{g mL}^{-1}$  chloramphenicol were inoculated from single HB101 *E. coli* colonies and grown overnight at 37°C with



agitation at 180 RPM. Expression plates were created by replicate plating of transformants into 170  $\mu\text{L}$  LB media with 35  $\mu\text{g mL}^{-1}$  chloramphenicol and grown at 37°C with agitation at 180 RPM for 8 hours. After 8 hours, expression plates were induced with 0.1 mM IPTG and incubated at 17°C and with agitation at 180 RPM for 18 hours. After 18 hours, osmotic shock was performed in 96-well microtiter plates by centrifugation at 1500 RPM (1230 x g) to obtain cell pellets; re-suspension of cell pellets in 5X volume of chilled osmotic shock buffer composed of 20% (w/v) sucrose, 100 mM TrisHCl and 10 mM EDTA at pH 8.0; centrifugation at 1500 RPM (1230 x g) to obtain cell pellets; re-suspension in 5X volume of chilled 1 mM EDTA to release the periplasmic fraction; and centrifugation at 1500 RPM (1230 x g) to pellet cell debris. Master plates were stored at -80°C after addition of 50  $\mu\text{L}$  of 70% sterile glycerol. Large-scale expression and osmotic shock to obtain periplasmic extracts of library hits was completed as described in Chapter 3.

#### *4.2.5 Ampicillin Synthesis Reactions with Racemic and Pure Substrates*

Periplasmic extracts obtained from 96-well microtiter expression plates were utilized in reactions containing 90 mM *rac*-PGME and 20 mM 6-APA in 100 mM phosphate buffer to complete the library screening. Reactions were started by the addition of 50  $\mu\text{L}$  of substrate mixture at 25°C and pH 7.0 to 10  $\mu\text{L}$  of periplasmic extract containing PGA variants in 96-well microtiter plates. After 6 hours the reactions were sampled and quenched by diluting 10-fold with the addition of HPLC eluent for analysis with HPLC. Library hits were expressed on a large-scale and their periplasmic extracts were used for reactions containing 90 mM *rac*-PGME and 20 mM 6-APA; 60 mM (*R*)-

phenylglycine methyl ester and 20 mM 6-APA; and 60 mM (*S*)-phenylglycine methyl ester and 20 mM 6-APA. All larger scale reactions were in 100 mM phosphate buffer, at 25°C and pH 7.0, contained 37 µg of active PGA variant, and had a total volume of 1600 µL. Reaction samples were taken at various points during the reaction and were quenched by diluting 10-fold with the addition of HPLC eluent for analysis with HPLC.

#### *4.2.6 High Performance Liquid Chromatography*

High-performance liquid chromatography (HPLC) analysis was completed with a Shimadzu-LC-20AT pump, Beckman Coulter Ultrasphere ODS 4.6 mm x 25 cm column, and SPD-M20A prominence diode array detector monitored at 215 nm. The mobile phase was isocratic at 1.0 mL min<sup>-1</sup> that initially contained 12.5% methanol and 87.5% 20 mM phosphate buffer at pH 7.0, increased via a step change to 30.0% methanol and 70% 20 mM phosphate buffer at pH 7.0 after 7 minutes, and decreased via a step change to 12.5% methanol and 87.5% 20 mM phosphate buffer at pH 7.0 after 30 min until 40 min. Phenylglycine, 6-APA, (*R*)-ampicillin, and (*S*)-ampicillin were analyzed on the HPLC. (*R*)-ampicillin and (*S*)-ampicillin diastereomers separate on the HPLC, however, the enantiomers of (*R*)-phenylglycine and (*S*)-phenylglycine; and (*R*)-phenylglycine methyl ester and (*S*)-phenylglycine methyl ester co-eluted with the stated method. See Appendix B for more information on analytical methods.

#### *4.2.7 Chromogenic Activity Assay with 2-Nitro-5-[(Phenylacetyl)amino]-Benzoic Acid*

2-Nitro-5-[(phenylacetyl)amino]-benzoic acid (NIPAB), a chromogenic substrate and a penicillin G analog, was employed in order to determine the hydrolysis activity of

the PGA variants. The hydrolysis of NIPAB liberates 5-amino-2-nitrobenzoic acid, which was monitored at 405 nm with a Beckmann-Coulter DU®800 spectrophotometer. NIPAB hydrolysis reactions were carried out at 25°C in 50 mM phosphate buffer at pH 7.5 [9]. Detailed information for the NIPAB assay, along with other analytical techniques can be found in Appendix B.

#### *4.2.8 Active Site Titration with Phenylmethanesulfonyl Fluoride*

The amount of active PGA was determined by duplicate active site titrations with the irreversible inhibitor phenylmethanesulfonyl fluoride (PMSF). Wild-type PGA and PGA variants were incubated with varying concentrations of PMSF for at least 10 minutes and residual activity on NIPAB was measured. Varying concentrations of PMSF were prepared for immediate use by diluting a 10 mM stock solution of PMSF in acetonitrile with 50 mM phosphate buffer at pH 7.5 [9].

### **4.3 Results and Discussion**

#### *4.3.1 Screening for Mutants with Improved Selectivity*

Diverse NNK libraries were screened by comparing the ratio of the production of (*R*)-ampicillin to (*S*)-ampicillin for PGA variants to that of the wild-type PGA in the synthesis of ampicillin from *rac*-PGME and 6-APA in 96-well microtiter plates. PGA libraries targeting improvements in the synthesis of semi-synthetic  $\beta$ -lactam antibiotics are typically difficult to screen due to the combination of varying expression and activity levels among variants, and to the transient nature of antibiotic formation observed in synthesis reactions. As a result, it is often difficult to determine an appropriate sampling

time when screening PGA variants for improved synthesis properties. However, these difficulties are all but eliminated when screening variants for improved selectivity, as selectivity at an early point in the reaction is typically indicative of the selectivity observed during the course of the reaction. Furthermore, screening for improved mutants with the desired synthesis reaction, rather than selecting for active mutants with the widely-employed auxotrophic hydrolysis based assay with D-phenylglycyl-L-leucine, proved to be necessary for this study, as the majority of our improved variants showed significantly decreased hydrolysis activity.

We screened at least 94 PGA variants which results in greater than 95% library coverage for each NNK library. Of all the PGA variants screened, 23% were active variants and 76% were inactive variants, agreeing with what is expected when making mutations around the active site of PGA [10]. PGA is expressed as a single cytoplasmic precursor and must undergo autocatalytic processing to render an active enzyme. The residues in the active site of PGA are notoriously difficult to mutate due to their participation in precursor processing [11]. Of the active variants, 14% had improved selectivity towards (*R*)-ampicillin synthesis and 59% had improved selectivity for (*S*)-ampicillin synthesis, though many of the variants showed low activity or poor expression. The remaining active variants did not show a significant change in diastereomer selectivity. As we are ultimately interested in obtaining an (*R*)-selective PGA, all variants that showed improvement towards (*R*)-ampicillin selectivity were sequenced. Additionally, the top 25 variants that showed improvement towards (*S*)-ampicillin selectivity were sequenced.

Overall, sequencing revealed thirteen non-duplicitous variants from our library screening as potentially interesting mutants for improving the selectivity of PGA. Three variants showed improvement towards (*R*)-ampicillin synthesis and 10 variants showed improvements for (*S*)-ampicillin synthesis, as assayed by our 96-well microtiter plate screening reactions.  $\beta$ Phe24Ala was not identified from library screening, but was targeted for mutagenesis due to the fact that it was previously shown to be selective for (*R*)-enantiomers with substituents other than an amino group at the C $_{\alpha}$  position [7]. This point mutation proved to be very difficult to obtain which is likely why it was not captured during the library screening. All fourteen variants were expressed on a large-scale and the purities of their periplasmic extracts were determined with duplicate phenylmethylsulfonyl fluoride (PMSF) titrations. The purities of periplasmic extracts for the PGA variant ranged from 10% to 25% and were confirmed with SDS-PAGE analysis. Subsequently, large-scale ampicillin synthesis reactions with *rac*-PGME were completed in order to confirm the selective behavior of the PGA variants observed in the 96-well microtiter plate screen.

#### 4.3.2 Ampicillin Synthesis from Racemic Substrates

Large-scale synthesis reactions from 90 mM *rac*-PGME and 20 mM 6-APA were carried out over a 10 hour time period, with samples taken at least every hour. The results from these reactions are shown in Table 4.1. From these reactions we were able to confirm that the three variants that showed improved (*R*)-selectivity as assayed by the well-plate screen, variants  $\beta$ Phe24Cys,  $\beta$ Phe24Pro, and  $\beta$ Phe24Ser, do in fact show improved selectivity towards (*R*)-ampicillin synthesis. Additionally, we discovered that

variant  $\beta$ Phe24Ala also shows increased selectivity towards (*R*)-ampicillin synthesis. Furthermore, we were able to confirm that four of the variants that showed improved (*S*)-selectivity as assayed by the well-plate screen, variants  $\alpha$ Phe146Ala,  $\alpha$ Phe146Arg,  $\alpha$ Phe146Gln,  $\alpha$ Phe146Tyr, show improved selectivity towards (*S*)-ampicillin. Variant  $\alpha$ Phe146Thr showed unexpected behavior, as the selectivity is conversion-dependent. The remaining variants identified by our library screening effort, which include  $\alpha$ Phe146Lys,  $\beta$ Pro22Asn,  $\beta$ Pro22Leu, and  $\beta$ Thr68Ser, exhibited either very low activity or low selectivity and were not studied further. Ampicillin conversion profiles for wild-type PGA, the most (*R*)-selective variant, the most (*S*)-selective variant, and for the variant that exhibits conversion-dependent selectivity is shown in Figure 4.3.

Perhaps the most obvious trend observed in selective mutants is that all variants that have improved selectivity for (*R*)-ampicillin synthesis result from an amino acid change at the  $\beta$ 24 position and all variants that have improved selectivity for (*S*)-ampicillin synthesis result from an amino acid change at the  $\alpha$ 146 position. The crystal structure of wild-type PGA complexed with (*R*)- $\alpha$ -methyl phenylacetic acid (PDB 1K79) shows that  $\beta$ Phe24 and  $\alpha$ Phe146 are on opposite sides of the substrate's  $C\alpha$ , so it is consistent to expect that changes in these positions could lead to enhancements in selectivity in opposite directions.

Table 4.1: Results from ampicillin synthesis reactions starting from 90 mM *rac*-PGME and 20 mM 6-APA resulting in the production of (*R*)-ampicillin ((*R*)-AMP) and (*S*)-ampicillin ((*S*)-AMP).

	d.e. <sub>R</sub> <sup>[a]</sup>	d.e. <sub>S</sub> <sup>[a]</sup>	Initial Rate <sup>[b]</sup> ( <i>R</i> )-AMP	Relative Rate ( <i>R</i> )-AMP	Initial Rate <sup>[b]</sup> ( <i>S</i> )-AMP	Relative Rate ( <i>S</i> )-AMP	Initial Rate <sup>[b]</sup> Total	Relative Rate Total	Maximum Yield ( <i>R</i> )-AMP	Relative Yield ( <i>R</i> )-AMP	Maximum Yield ( <i>S</i> )-AMP	Relative Yield ( <i>S</i> )-AMP	Maximum Yield Total	Relative Yield Total
WT PGA	37%	-	0.086	1.00	0.048	1.00	0.135	1.00	21.0%	1.00	9.7%	1.00	30.7%	1.00
<i>Variants Selective for (R)-Ampicillin Synthesis</i>														
βPhe24Ala	98%	-	0.082	0.95	2.15E-04	0.00	0.082	0.61	42.4%	2.02	0.4%	0.04	42.8%	1.39
βPhe24Ser	97%	-	0.036	0.42	2.37E-04	0.00	0.036	0.27	35.0%	1.66	0.5%	0.05	35.5%	1.15
βPhe24Pro	95%	-	0.018	0.20	4.58E-04	0.01	0.018	0.13	26.5%	1.26	0.7%	0.07	27.2%	0.88
βPhe24Cys	70%	-	0.007	0.08	1.60E-03	0.03	0.009	0.06	15.0%	0.71	2.7%	0.28	17.7%	0.57
<i>Variants Selective for (S)-Ampicillin Synthesis</i>														
αPhe146Ala	-	51%	0.002	0.03	0.036	0.75	0.038	0.28	3.3%	0.16	10.2%	1.05	13.5%	0.44
αPhe146Gln	-	57%	1.06E-03	0.01	0.006	0.12	0.007	0.05	2.8%	0.14	10.3%	1.06	13.2%	0.43
αPhe146Arg	-	65%	5.76E-04	0.01	0.004	0.08	0.004	0.03	1.8%	0.09	8.6%	0.88	10.4%	0.34
αPhe146Tyr	-	36%	0.003	0.04	3.26E-03	0.07	0.006	0.05	1.7%	0.08	3.5%	0.36	5.1%	0.17
<i>Variants that Exhibit Conversion-Dependent Selective Behavior</i>														
αPhe146Thr	-	-	0.043	0.49	0.116	2.40	0.158	1.17	34.1%	1.62	2.2%	0.22	36.3%	1.18

[a] d.e. reported at maximum (*R*)-ampicillin concentration. [b] Rate reported is in mM min<sup>-1</sup>.

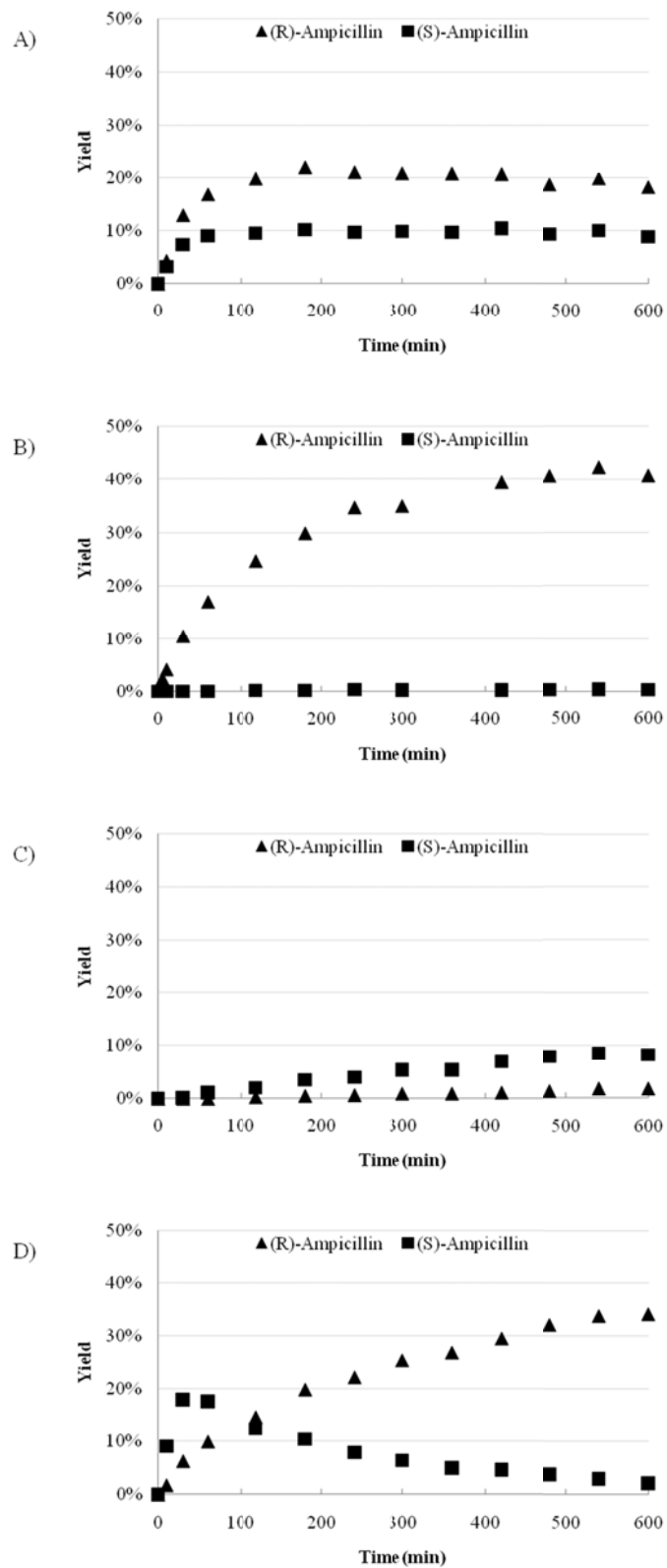


Figure 4.3: Ampicillin synthesis from 20 mM 6-APA and 90 mM *rac*-PGME for A) wild-type PGA, B) PGA variant  $\beta$ Phe24Ala, C) PGA variant  $\alpha$ Phe146Arg, and D) PGA variant  $\alpha$ Phe146Thr



#### 4.3.2.1 Variants Selective for (*R*)-Ampicillin Synthesis

Crystal structures of wild-type PGA and  $\beta$ Phe24Ala complexed with (*R*)- $\alpha$ -methyl phenylacetic acid (PDB 1K79 and 1K5S, respectively) show that the mutation does not result in significant positional rearrangement of the catalytic residues or the loop containing the  $\beta$ 24 residue. Furthermore, the two crystal structures show that the removal of a phenyl ring creates space in the binding pocket to better accommodate  $C_\alpha$ -substituted (*R*)-enantiomers in comparison to wild-type PGA, explaining the 10-fold higher binding affinities observed by Alkema et al. for variant  $\beta$ Phe24Ala as compared to those observed for wild-type PGA for  $C_\alpha$ -substituted substrates [7]. It is reasonable to assume that alanine, serine, and cysteine would have similar structural impacts. That being said, the d.e.<sub>R</sub> values reported in Table 4.1 for  $\beta$ Phe24Ala,  $\beta$ Phe24Ser and  $\beta$ Phe24Cys correlate directly with the sizes of the amino acids present in each variant. Alanine occupies the least amount of space, 88.6 Å<sup>3</sup> [12], and is the most selective variant with a d.e.<sub>R</sub> value of 98%. Serine is not much larger; it occupies a volume of 89.0 Å<sup>3</sup> [12], and is only slightly less selective with a d.e.<sub>R</sub> value of 97%. Cysteine is considerably larger; it occupies a volume of 117.7 Å<sup>3</sup> [12], and as a result is less selective with a d.e.<sub>R</sub> value of 70%. Variant  $\beta$ Phe24Pro does not follow this trend, though inserting a proline would affect the backbone structure of the protein, likely kinking the loop containing residue  $\beta$ 24 and creating additional space in the binding pocket. This would explain why  $\beta$ Phe24Pro has a d.e.<sub>R</sub> value of 95% despite occupying 122.7 Å<sup>3</sup> of space [12]. Furthermore, the crystal structures of variant  $\beta$ Phe24Ala show that when a substituent on the  $C_\alpha$  is present, the enzyme occupies the closed confirmation where  $\alpha$ Phe146 shifts and covers the binding pocket [7]. As a result,  $\alpha$ Phe146 occupies space that (*S*)-substituents would occupy,

explaining why  $\beta$ Phe24Ala,  $\beta$ Phe24Ser,  $\beta$ Phe24Pro, and  $\beta$ Phe24Cys, show significantly decreased (*S*)-ampicillin synthetic activity.

#### 4.3.2.2 Variants Selective for (*S*)-Ampicillin Synthesis

The amino acid substitutions at the  $\alpha$ 146 position that lead to an increase in selectivity for (*S*)-ampicillin synthesis are all very different. As shown in Table 4.1, the most selective variant is  $\alpha$ Phe146Arg with a d.e.<sub>S</sub> value of 65%, followed by  $\alpha$ Phe146Gln with a d.e.<sub>S</sub> value of 57%,  $\alpha$ Phe146Ala with a d.e.<sub>S</sub> value of 51%, and  $\alpha$ Phe146Tyr with a d.e.<sub>S</sub> value of 36%. Obviously, there is no trend in the observed selectivity of these variants with polarity, charge, or size of the amino acids, rendering it difficult to ascertain what factors are contributing to the observed increase in selectivity. Similarly, Jager et al. found it difficult to correlate properties of the amino acids introduced at the  $\alpha$ 146 position to their effects on the kinetic properties of PGA [13]. However, it is well known from crystal structures of PGA that the residue at the  $\alpha$ 146 position is flexible and can adopt multiple energetically favored conformations. In the wild-type enzyme, the movement of  $\alpha$ Phe146 is thought to occur either to create space in the binding pocket to accommodate larger substrates or to create additional binding interactions with the substrate, depending on the situation [7]. A crystal structure for  $\alpha$ Phe146Tyr complexed with (*R*)- $\alpha$ -methyl phenylacetic acid shows no conformational changes in comparison to the wild-type, but because the position of  $\alpha$ Phe146 is situational no certain conclusions can be drawn from it [7]. The variants that show increased selectivity for (*S*)-ampicillin synthesis are likely those that are able to adopt energetically favorable conformations that accommodate the (*S*)-substituent.

#### 4.3.2.3 Variants that Exhibit Conversion-Dependent Selective Behavior

Variant  $\alpha$ Phe146Thr shows interesting behavior as its observed selectivity is dependent on the extent of the reaction. Initially the variant is selective for (*S*)-ampicillin synthesis with an initial d.e.<sub>S</sub> value of 67% at 10% yield. However, the resulting (*S*)-ampicillin produced by the enzyme is consumed by secondary hydrolysis while (*R*)-ampicillin synthesis proceeds, resulting in a switch in selectivity and a d.e.<sub>R</sub> value of 88% at 36% yield. This phenomenon is thought to be the result of altering the enzyme's inhibition for either (*S*)-phenylglycine ((*S*)-PG) or (*S*)-ampicillin. Although not confirmed, examining the reaction profiles that result from reactions with pure (*R*)-PGME, pure (*S*)-PGME and *rac*-PGME has led us to hypothesize that wild-type PGA is inhibited by either (*S*)-ampicillin or (*S*)-PG. This could explain why very little secondary hydrolysis of (*S*)-ampicillin is observed in reactions with the wild-type as compared to the  $\alpha$ Phe146Thr variant. Inhibition studies for (*S*)-ampicillin and (*S*)-PG with wild-type PGA and  $\alpha$ Phe146Thr would need to be completed to confirm this hypothesis.

#### *4.3.3 Ampicillin Synthesis from Pure Substrates*

The nine interesting mutants were further characterized by investigating their ability to synthesize (*R*)-ampicillin and (*S*)-ampicillin from pure substrates over a 24 hour time period, with samples taken at least every two hours for the first 12 hours. The results from the ampicillin synthesis reactions starting with 20 mM 6-APA and 60 mM (*R*)-phenylglycine methyl ester ((*R*)-PGME) are shown in Table 4.2 and Figure 4.4. The results for ampicillin synthesis reactions starting from 20 mM 6-APA and 60 mM (*S*)-phenylglycine methyl ester ((*S*)-PGME) are shown in Table 4.3 and Figure 4.5.

Table 4.2: (R)-ampicillin ((R)-AMP) synthesis from 20 mM 6-APA and 60 mM (R)-PGME

	Initial Rate <sup>[a]</sup>	Relative Rate	Initial Rate <sup>[a]</sup>	Relative Rate	S/H- ratio	Maximum Yield	Relative Yield
	(R)-PG	(R)-PG	(R)-AMP	(R)-AMP		(R)-AMP	(R)-AMP
WT PGA	0.325	1.00	0.256	1.00	0.79	38.2%	1.00
<i>Variants Selective for (R)-Ampicillin Synthesis</i>							
βPhe24Ala	0.083	0.26	0.278	1.09	3.35	85.7%	2.24
βPhe24Ser	0.065	0.20	0.133	0.52	2.04	82.7%	2.17
βPhe24Pro	0.065	0.20	0.127	0.50	1.95	74.4%	1.95
βPhe24Cys	0.033	0.10	0.050	0.19	1.49	42.7%	1.12
<i>Variants Selective for (S)-Ampicillin Synthesis</i>							
αPhe146Ala	0.046	0.14	0.015	0.06	0.33	35.7%	0.93
αPhe146Gln	0.039	0.12	0.004	0.02	0.11	16.2%	0.42
αPhe146Arg	0.039	0.12	0.002	0.01	0.06	9.6%	0.25
αPhe146Tyr	0.360	1.11	0.005	0.02	0.01	3.4%	0.09
<i>Variants that Exhibit Conversion-Dependent Selective Behavior</i>							
αPhe146Thr	0.102	0.31	0.172	0.67	1.69	80.5%	2.11

[a] Rate reported is in mM min<sup>-1</sup>.

Table 4.3: (S)-ampicillin ((S)-AMP) synthesis from 20 mM 6-APA and 60 mM (R)-PGME

	Initial Rate <sup>[a]</sup>	Relative Rate	Initial Rate <sup>[a]</sup>	Relative Rate	S/H- ratio	Maximum Yield	Relative Yield
	(S)-PG	(S)-PG	(S)-AMP	(S)-AMP		(S)-AMP	(S)-AMP
WT PGA	0.073	1.00	0.090	1.00	1.23	18.5%	1.00
<i>Variants Selective for (R)-Ampicillin Synthesis</i>							
βPhe24Ala	0.033	0.45	9.61E-04	0.01	0.03	2.6%	0.14
βPhe24Ser	0.034	0.46	6.64E-04	0.01	0.02	1.6%	0.09
βPhe24Pro	0.036	0.49	1.09E-03	0.01	0.03	2.4%	0.13
βPhe24Cys	0.044	0.60	2.28E-03	0.03	0.05	3.4%	0.18
<i>Variants Selective for (S)-Ampicillin Synthesis</i>							
αPhe146Ala	0.077	1.05	0.066	0.74	0.86	14.9%	0.80
αPhe146Gln	0.053	0.72	0.015	0.16	0.28	13.6%	0.73
αPhe146Arg	0.046	0.63	0.009	0.10	0.19	11.8%	0.64
αPhe146Tyr	0.085	1.16	0.010	0.11	0.12	6.1%	0.33
<i>Variants that Exhibit Conversion-Dependent Selective Behavior</i>							
αPhe146Thr	0.125	1.72	0.276	3.08	2.20	21.2%	1.15

[a] Rate reported is in mM min<sup>-1</sup>.

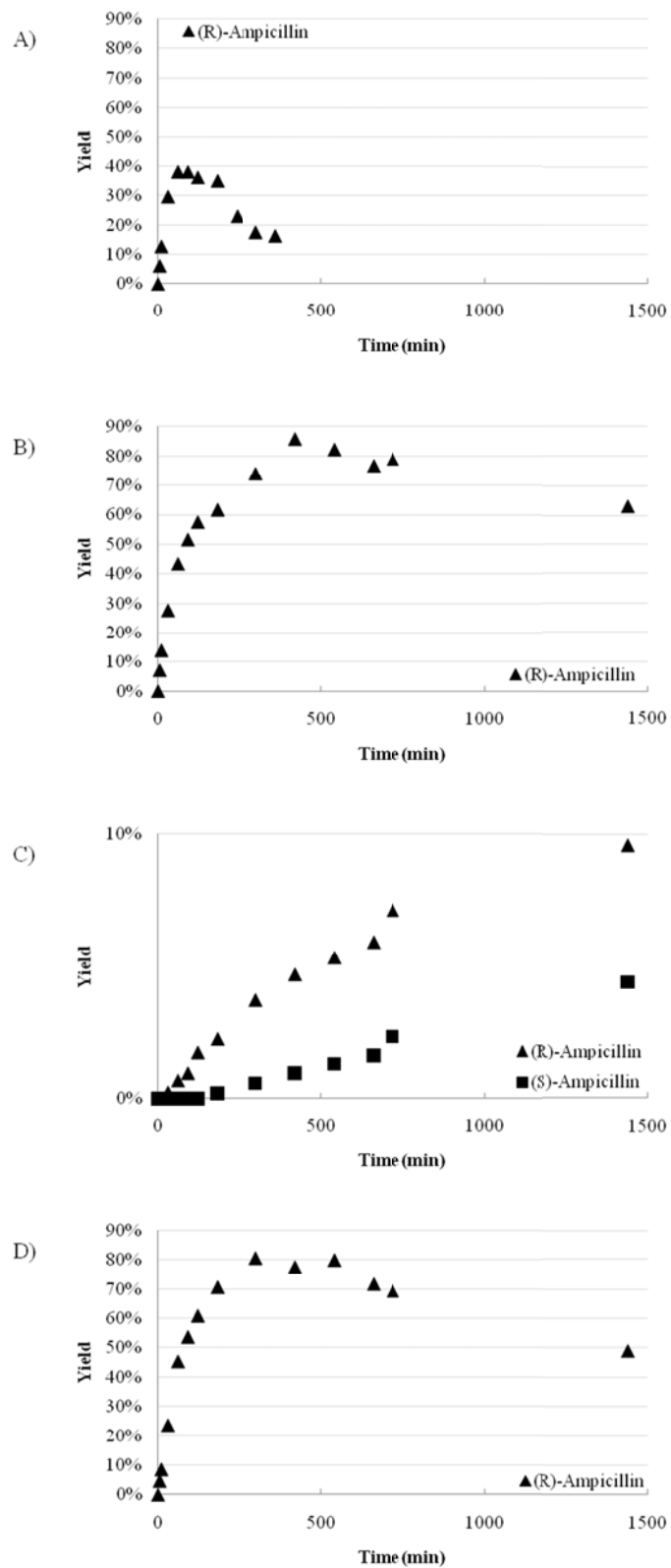


Figure 4.4: Ampicillin synthesis from 20 mM 6-APA and 60 mM (R)-PGME for A) wild-type PGA, B) PGA variant  $\beta$ Phe24Ala, C) PGA variant  $\alpha$ Phe146Arg, and D) PGA variant  $\alpha$ Phe146Thr

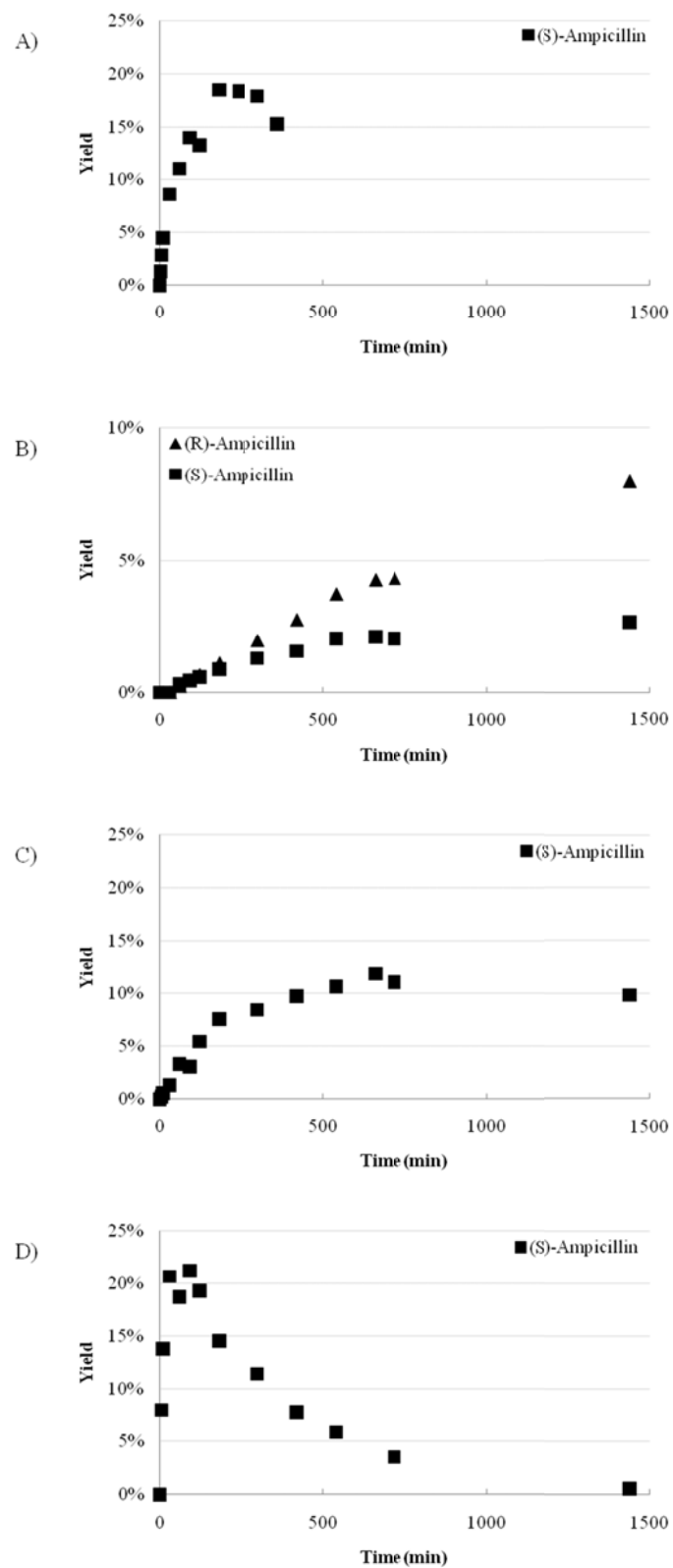


Figure 4.5: Ampicillin synthesis from 20 mM 6-APA and 60 mM (S)-PGME for A) wild-type PGA, B) PGA variant  $\beta$ Phe24Ala, C) PGA variant  $\alpha$ Phe146Arg, and D) PGA variant  $\alpha$ Phe146Thr

#### 4.3.3.1 Variants Selective for (*R*)-Ampicillin Synthesis

The (*R*)-selective variants that we have identified have proven to not only be more selective, but also lead to an enhancement in the yield achieved when synthesizing ampicillin from pure (*R*)-PGME as shown in Table 4.2. The synthesis with variant  $\beta$ Phe24Ala results in the highest yield, 85.7%, and has increased synthetic activity over wild-type PGA by a factor of 1.09. These results agree with the 3-fold increase in yield and the equivalent activity observed during ampicillin synthesis for  $\beta$ Phe24Ala in comparison to wild-type PGA that has been reported by Alkema et al. and the 4-fold increase in yield observed by Gabor et al. for a  $\beta$ Phe24Ala variant from penicillin acylase PAS2 [10, 14]. Variant  $\beta$ Phe24Ser has decreased synthetic activity in comparison to the wild-type PGA by a factor of 0.52, but is ultimately still able to achieve a yield of 82.7%. Similarly,  $\beta$ Phe24Pro has decreased synthetic activity in comparison to the wild-type PGA by a factor of 0.50 but yields 74.4% of ampicillin. Finally,  $\beta$ Phe24Cys shows decreased synthetic activity by a factor of 0.19 in comparison the wild-type but still achieves a yield of 42.7%. The trend in our results are similar to those reported when You et al. site-saturated  $\beta$ Phe24 and found the synthetic activity for  $\beta$ Phe24Ala to be 330% relative to wild-type PGA,  $\beta$ Phe24Ser to be 23%,  $\beta$ Phe24Pro to be 31%, and  $\beta$ Phe24Cys to be 0% for cefadroxil synthesis from (*R*)-p-phenylglycine hydroxy ethyl ester and 7-aminodeacetoxycephalosporanic acid [15]. Closer examination of the data shows that all of the (*R*)-selective variants have significantly decreased hydrolytic activity. As a result, we observe notably increased ratios of the synthesis of ampicillin to the generation of (*R*)-phenylglycine ((*R*)-PG) from primary hydrolysis, also known the synthesis to hydrolysis ratio (S/H-ratio), for the (*R*)-selective PGA variants as compared

to wild-type PGA. An increase in the S/H-ratio is known to increase the yield in the kinetically-controlled synthesis of ampicillin, explaining why the (*R*)-selective variants show an improvement in yield despite decreases in their synthetic activity. Also contributing to the yield enhancement is the considerable decrease in secondary hydrolysis observed, which is shown in Figure 4.4. The work by Alkema et al. supports these findings for  $\beta$ Phe24Ala as they report a 4-fold decrease in  $k_{\text{cat}}/K_M$  for ampicillin in comparison to wild-type PGA and the 20-fold decrease in  $k_{\text{cat}}/K_M$  for ampicillin with a  $\beta$ Phe24Ala variant from penicillin acylase PAS2 [10, 14].

The (*R*)-selective variants result in a significantly decreased hydrolytic activity, synthetic activity, and yield when synthesizing ampicillin from (*S*)-PGME as shown in Table 4.3. The most interesting result from this set of experiments is that the (*R*)-selective variants actually produced (*R*)-ampicillin when starting from pure (*S*)-PGME. After 24 hours, variant  $\beta$ Phe24Ala yielded 8.0% (*R*)-ampicillin,  $\beta$ Phe24Ser yielded 8.3% (*R*)-ampicillin,  $\beta$ Phe24Pro yielded 6.6% (*R*)-ampicillin, and  $\beta$ Phe24Cys yielded (*R*)-ampicillin. As shown in Figure 4.5, the rate that  $\beta$ Phe24Ala catalyzes (*R*)-ampicillin synthesis is 1.5-fold faster than the rate that it catalyzes (*S*)-ampicillin synthesis, though (*R*)-ampicillin synthesis does not begin until 27 minutes after the reaction was initiated. Similarly,  $\beta$ Phe24Ser catalyzes (*R*)-ampicillin synthesis at 2.2 times the rate of (*S*)-ampicillin, but (*R*)-ampicillin synthesis does not begin until 69 minutes after the reaction was initiated. As our analytical techniques only separate diastereomers, it is unknown if these variants are acting as a racemases, epimerases, or both. This type of behavior is only observed in the wild-type enzyme on long time scales and even then yields of (*R*)-ampicillin are less than 1%. Because we've never seen this type of behavior with the



wild-type enzyme on the time-scales we employ, we can also rule out the possibility that the racemization is occurring chemically. This intriguing phenomenon is not understood and to our knowledge has never been reported for wild-type PGA or any PGA variants.

#### 4.3.3.2 Variants Selective for (*S*)-Ampicillin Synthesis

The (*S*)-selective variants that we have identified all show a decrease in hydrolytic activity, synthetic activity and yield achieved when synthesizing ampicillin from pure (*R*)-PGME and 6-APA. However, similar to how the (*R*)-selective variants are able to catalyze (*R*)-ampicillin synthesis from (*S*)-PGME, the (*S*)-selective variants are able to catalyze (*S*)-ampicillin synthesis from pure (*R*)-PGME. Variant  $\alpha$ Phe146Ala yielded 1.5% (*S*)-ampicillin,  $\alpha$ Phe146Gln yielded 5.4% (*S*)-ampicillin,  $\alpha$ Phe146Arg yielded 4.4% (*S*)-ampicillin, and  $\alpha$ Phe146Tyr yielded less than 1% (*S*)-ampicillin. The (*S*)-selective variants catalyze (*S*)-ampicillin synthesis at much slower rates than they synthesize (*R*)-ampicillin when starting from pure (*R*)-PGME, nonetheless the fact that these PGA variants can act as a racemase or epimerase is fascinating.

The (*S*)-selective variants also show a decrease in synthetic activity and yield in the synthesis of ampicillin from (*S*)-PGME and 6-APA compared to wild-type PGA. As discussed above, the S/H-ratio is an indicator of the overall yield in the kinetically-controlled synthesis of ampicillin, and the (*S*)-selective variants have a decreased S/H-ratio for all synthesis reactions from pure substrates. This is in contrast to previous reports that  $\alpha$ Phe146Ala has a two-fold increase in (S/H)-ratio but activity so low that a reliable concentration for ampicillin could not be determined in the synthesis from 15 mM (*R*)-PGA and 30 mM 6-APA. Furthermore, these results differ from those reported

by Jager et al. for ampicillin synthesis from 15 mM (*R*)-phenylglycine amide ((*R*)-PGA) and 10 mM 6-APA. Jager et al. site-saturated  $\alpha$ Phe146 and found that  $\alpha$ Phe146Ala achieved 99% of the maximum yield of wild-type PGA,  $\alpha$ Phe146Gln achieved 114%,  $\alpha$ Phe146Arg achieved 3% and  $\alpha$ Phe146Tyr achieved 0% for ampicillin synthesis from 15 mM (*R*)-PGA and 10 mM 6-APA [13]. This clearly corroborates previous reports that esterase and amidase activity are not necessarily equivalent [14]. The most drastic decrease in S/H-ratio is observed for  $\alpha$ Phe146Tyr, which has decreased nearly 60-fold in the synthesis from (*R*)-PGME and decreased 10-fold in the synthesis from (*S*)-PGME, agreeing with previously reported results of a 40-fold decrease in S/H-ratio for the synthesis of ampicillin from (*R*)-PGA and 6-APA [14]. In all cases, the decrease in the S/H-ratio can be explained by the fact that  $\alpha$ Phe146 is known to have van der Waals interactions with the  $\beta$ -lactam nucleus. Therefore mutating this residue will decrease the affinity of the enzyme for 6-APA and thereby decrease the synthesis activity of the enzyme.

#### 4.3.3.3 Variants that Exhibit Conversion-Dependent Selective Behavior

$\alpha$ Phe146Thr shows an increase in yield for (*R*)-ampicillin synthesis starting from pure (*R*)-PGME and an increase in yield for (*S*)-ampicillin synthesis starting from pure (*S*)-PGME. For the reactions starting from 6-APA and pure (*R*)-PGME, the variant shows decreased synthetic activity by a factor of 0.67 and decreased hydrolytic activity of 0.31, leading to an improved S/H-ratio and therefore a yield increase to 80.5%. In contrast, for the reaction starting from 6-APA and pure (*S*)-PGME,  $\alpha$ Phe146Thr shows increased hydrolytic activity by a factor of 1.72 and increased synthetic activity by a

factor of 3.08, also resulting in an increase in the synthesis to hydrolysis activity and an improved yield of 21.2% (S)-ampicillin. An improvement in S/H-ratio and yield has been previously reported for  $\alpha$ Phe146Thr [13].  $\alpha$ Phe146Thr exhibits racemase or epimerase activity, though in yields less than 1% for both reactions from pure substrates.

Table 4.4: NIPAB hydrolysis by PGA variants

	$V_{\max}$ ( $\mu\text{mol NABA/min mg PGA}$ )	Relative $V_{\max}$	$K_M$ ( $\mu\text{M}$ )	Relative $K_M$
WT PGA	17.3	1.00	26.0	1.00
<i>Variants Selective for (R)-Ampicillin Synthesis</i>				
$\beta$ Phe24Ala	1.0	0.06	98.2	3.77
$\beta$ Phe24Ser	0.6	0.04	550.9	21.18
$\beta$ Phe24Pro	2.9	0.17	796.5	30.61
$\beta$ Phe24Cys	ND <sup>[a]</sup>	ND	ND	ND
<i>Variants Selective for (S)-Ampicillin Synthesis</i>				
$\alpha$ Phe146Ala	7.3E-02	4.2E-03	115.9	4.45
$\alpha$ Phe146Gln	4.9E-02	2.8E-03	271.7	10.44
$\alpha$ Phe146Arg	5.3E-02	3.1E-03	386.2	14.84
$\alpha$ Phe146Tyr	1.3	0.08	0.5	0.02
<i>Variants that Exhibit Conversion-Dependent Selective Behavior</i>				
$\alpha$ Phe146Thr	2.5E-01	0.01	78.5	3.02

[a] Not determined, not able to obtain repeatable data.

#### 4.3.4 2-Nitro-5-[(Phenylacetyl)amino]-Benzoic Acid Hydrolysis

All nine mutants were further characterized by investigating their ability to hydrolyze 2-nitro-5-[(phenylacetyl)amino]-benzoic acid (NIPAB) and the results are shown in Table 4.4. NIPAB is a chromogenic analog of penicillin G and is widely used to characterize the hydrolysis activity of PGA variants. With the exception of  $\alpha$ Phe146Tyr, all of the variants had significantly decreased hydrolytic activity and binding affinities for NIPAB as evidenced by hugely increased  $K_M$  values. This agrees with the results that Alkema et al. presented for variants  $\beta$ Phe24Ala,  $\alpha$ Phe146Ala,  $\alpha$ Phe146Tyr, and supports the hypothesis that the removal of a phenyl ring from the

active site of PGA decreases the binding affinity for its substrates [4, 14]. Previous reports indicate that only C $_{\alpha}$ -substituted substrates bind tightly to  $\beta$ Phe24Ala [7]. The amino acid variation at  $\beta$ Phe24Ser and  $\beta$ Phe24Cys are assumed to have structural impacts equivalent to  $\beta$ Phe24Ala and therefore the lack of a C $_{\alpha}$  substituent would contribute to their low binding affinities for NIPAB as well.

#### 4.4 Conclusions

In this work, we succeeded in altering the diastereomeric selectivity of PGA by site-saturating five residues in the active site. Four PGA variants were found to have improved selectivity for (*S*)-ampicillin synthesis, all resulting from an amino acid substitution at the  $\alpha$ 146 position. The position of the residue at  $\alpha$ 146 is known to be flexible and depend on the substrate bound, and therefore it is difficult to ascertain what factors led to the observed (*S*)-selective behavior. In the wild-type enzyme  $\alpha$ Phe146 is known to interact with the  $\beta$ -lactam nucleus and therefore the synthetic activity of the variants was affected as evidence by the decreased S/H-ratio and a decrease yield for the synthesis (*R*)-ampicillin and (*S*)-ampicillin from (*R*)-PGME and (*S*)-PGME, respectively, for all mutants.

Finding a PGA variant capable of asymmetrically synthesizing (*R*)-ampicillin would enable the use of racemic activated acyl side chains in the synthesis of ampicillin, cephalexin and cefaclor. We have discovered four (*R*)-selective variants with improved diastereomeric selectivity for (*R*)-ampicillin synthesis. The result is an improvement from the d.e.<sub>R</sub> value of 37% for the wild-type enzyme to a d.e.<sub>R</sub> value of 98% for our most selective mutant,  $\beta$ Phe24Ala. All (*R*)-selective variants resulted from an amino acid

substitution at the  $\beta$ 24 position and the extent of selectivity was shown to be dependent on the size of the amino acid substituted. Additionally, the (*R*)-selective variants were shown to have up to 4-fold decreases in S/H-ratio and up to 2-fold increases in the yield achieved when synthesizing ampicillin from pure (*R*)-PGME.

Even if the initial activated acyl side chain of choice for ampicillin is (*R*)-PGA, these variants can prove to be useful in the synthesis of semi-synthetic  $\beta$ -lactam antibiotics. The production of pure (*R*)-PGA from *rac*-phenylglycine amide with L-aminopeptidase results in a maximum of 50% conversion to the desired (*R*)-PGA and an excess of the (*S*)-PG. (*S*)-PG can be racemized and resolved with diastereomeric salt crystallization, or alternatively it can be esterified and racemized in one step with the use of naphtha cracking catalyst zeolite H-USY [16]. A dynamic kinetic resolution by selective ammonolysis of *rac*-PGME with *Candida antarctica* lipase B coupled with racemization with an aromatic aldehyde to selectively produce (*R*)-PGA has been previously proposed but has not been implemented because it only achieves an enantiomeric excess of 85% [17]. Replacing the lipase with a selective PGA would eliminate the need for the ammonolysis step, as PGA could synthesize ampicillin directly from the methyl ester, and the dynamic kinetic resolution nature of the process would be retained.

#### **4.5 Publication Information**

The research presented in Chapter 4 of this dissertation was submitted to Protein Engineering Design and Selection by August 10<sup>th</sup>, 2011 for publication with Janna K. Blum and Andreas S. Bommarius as co-authors.

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## CHAPTER 5

### CONCLUSIONS AND RECOMMENDATIONS

#### 5.1 Summary and Conclusions

The work presented in this dissertation encompasses reaction engineering, medium engineering, and data-driven protein engineering, to improve the enzymatic synthesis of semi-synthetic  $\beta$ -lactam antibiotics. Our studies focused on the kinetically-controlled synthesis of ampicillin, though the results are likely applicable to the synthesis of amoxicillin, cephalexin, cefadroxil, cefaclor and cefprozil.

Reaction engineering was employed to demonstrate the feasibility of utilizing a cascade conversion involving two biocatalytic steps in fully aqueous medium to produce ampicillin. A cascade set-up presents a promising alternative to the current two-pot set-up which requires intermittent isolation of the intermediate, 6-aminopenicillanic acid (6-APA). In the first reaction, the  $\beta$ -lactam moiety 6-APA was produced from the thermodynamically-controlled hydrolysis of penicillin G with immobilized PGA. The byproduct from this reaction, phenylacetic acid (PAA), is a known inhibitor of PGA [1]. In the second reaction, ampicillin was produced from the kinetically-controlled coupling of 6-APA and (*R*)-phenylglycine methyl ester ((*R*)-PGME) catalyzed by either PGA or  $\alpha$ -amino ester hydrolase (AEH) [2]. AEHs are unique in their specificity for  $\alpha$ -amino groups on the acyl moiety and therefore do not catalyze the hydrolysis of penicillin G and are not inhibited by PAA [3]. In this work, two different reaction configurations and various relative enzyme loadings were studied.



In all reaction configurations the two-enzyme system that utilized PGA and AEH out-performed the one-enzyme system that utilized only PGA. Furthermore, it was determined that relative enzyme loadings had a large effect on the maximum yield achieved and the degree of secondary hydrolysis observed. In a one-pot, one-step configuration, a batch process, penicillin G, (R)-PGME, and all enzyme(s) were added to the reaction and the reaction was allowed to proceed. The optimum enzyme loading was determined to be 99.2 UPenG PGA and 4.4 UAmp AEH which resulted in a maximum yield of 39% achieved in 60 minutes. In a one-pot, two-step configuration, penicillin G and PGA were added to the reaction and the hydrolysis reaction was allowed to proceed. When the hydrolysis reaction was near completion, (R)-PGME and either AEH or PGA was added to the reaction. The optimum enzyme loading was determined to be 99.2 UPenG PGA and 4.4 UAmp AEH which resulted in a maximum yield of 46% after 150 minutes.

Medium engineering is studied in  $\beta$ -lactam antibiotic synthesis to suppress undesired primary and secondary hydrolysis reactions. Ampicillin synthesis reactions from 6-APA and (R)-PGME in the 30% (v/v) ethylene glycol revealed that ethylene glycol reacts with (R)-PGME to form (R)-phenylglycine hydroxyethyl ester ((R)-PGHEE). The formation of (R)-PGHEE has never been reported or studied. The present work investigates the synthesis of ampicillin from different acyl donors in systems containing ethylene glycol, ethanol and tert-butyl alcohol, to test the hypothesis that transesterification of a side chain acyl donor with a co-solvent can act as an in-situ mixed donor synthesis and can positively affect yield. The transesterification of (R)-PGME to (R)-PGHEE rather than hydrolysis of (R)-PGME to (R)-PG re-directs the consumption of

(R)-PGME to an intermediate that could be used to synthesize ampicillin, rather than to an unusable hydrolysis side product.

When (R)-PGME is used as the activated acyl side chain, we have observed that the ampicillin yield enhancement is dependent on the amount of (R)-PGME present in the reactions. When (R)-PGA is used at the activated acyl side chain, we have found that the use of ethylene glycol as a co-solvent has minimal effects on the yield of ampicillin achieved. Furthermore, we have synthesized (R)-PGHEE and shown that it can act as an acyl side chain donor for the synthesis of ampicillin. Based on these results, there is evidence that the synthesis of ampicillin from (R)-PGME and 6-APA with an ethylene glycol co-solvent acts as an in situ mixed donor synthesis, where both (R)-PGME and (R)-PGHEE are acyl side chain donors for the synthesis of ampicillin.

Protein engineering was utilized to improve the diastereomeric selectivity of PGA so that racemic acid derivatives can be used in  $\beta$ -lactam antibiotic synthesis. When starting with *rac*-PGME, PGA will catalyze the synthesis of the desired product, (R)-ampicillin, in addition to an undesired product, (S)-ampicillin, exhibiting only weak selectivity for the desired product. Using PGA crystal structures, we targeted residues for mutation based on the proximity to the substrate's chiral center.

We identified four variants with improved selectivity for (R)-ampicillin synthesis, all resulting from a mutation at the  $\beta$ 24 position. The most selective mutant improved the d.e.<sub>R</sub> value of 37% for the wild-type enzyme to a d.e.<sub>R</sub> value of 98% for our most selective mutant,  $\beta$ Phe24Ala. Also, we identified four variants with mutations at the  $\alpha$ 146 position that resulted in (S)-selective PGA variants.  $\beta$ Phe24 and  $\alpha$ Phe 146 are on opposite sides of the alpha carbon of PGME, and we have shown that altering these

residues results in enhanced selectivities in opposite directions. All variants that showed selectivity for (S)-ampicillin synthesis showed decreased synthetic activity for racemic pure substrates, likely as a result of increasing the synthesis to hydrolysis ratio by altering the interaction of  $\alpha$ Phe146 with the  $\beta$ -lactam nucleus. In contrast, the mutants that were selective for (R)-ampicillin showed significantly decreased primary and secondary hydrolysis when synthesizing ampicillin from pure (R)-PGME, resulting 4-fold decreases in S/H-ratio and up to 2-fold increases in the yield achieved. Finally, we have discovered that the PGA variants have racemase or epimerase activity, a fascinating phenomenon that has never been reported and should be studied further.

Overall, we have improved the enzymatic synthesis of semi-synthetic  $\beta$ -lactam antibiotics by extending the understanding and applications of the biocatalysts that are utilized to synthesize them. We have elucidated an in-situ mixed donor synthesis that is the result of ampicillin synthesis in the presence of select acyl side chain donors and suitable co-solvents. We have shown that AEH and PGA can be combined in a novel cascade synthesis to eliminate one of the isolation steps in the current manufacturing process. Finally, we have made significant progress towards developing a selective PGA that would allow the use of racemic substrates to produce diastereomerically pure  $\beta$ -lactam antibiotics and eliminate the need to use enantiomerically pure substrates for their synthesis. Eliminating isolation and purification steps represent significant improvements as it renders the current process more economically feasible and environmentally benign.

## 5.2 Recommendations

### 5.2.1 Further Improvements to Penicillin G Acylase Selectivity

In Chapter 4 of this dissertation we used data-driven protein engineering techniques to improve the diastereoselectivity of penicillin G acylase (PGA) for the synthesis of (*R*)-ampicillin from 6-aminopenicillanic acid (6-APA) and *rac*-phenylglycine methyl ester (*rac*-PGME). Specifically, we were successful in increasing the diastereomeric excess (d.e.) value in the (*R*)-direction from 37% that the wild-type PGA exhibits to a d.e.<sub>R</sub> value of 98% for the most selective PGA variant,  $\beta$ Phe24Ala. However, any presence of (*S*)-ampicillin in the final drug product would be considered an impurity and the United States Food and Drug Administration's requires that all organic impurities above 0.1% should be isolated and characterized with full toxicology studies [4]. Therefore, while these results represent significant progress, even further improvements to the selectivity of PGA should be investigated to improve the d.e.<sub>R</sub> value of PGA to greater than 99.8%.

#### 5.2.1.1 Continued Use of Data-Driven Protein Engineering Techniques

Given the success of utilizing data-driven techniques for the present work, we propose that data-driven protein engineering techniques should continue to be employed to further push PGA towards the desired selectivity. With an improved variant of PGA already in hand, the next protein engineering technique that should be exercised is iterative saturation mutagenesis (ISM). ISM aims to improve a protein for a desired characteristic incrementally, taking the best variant from site-saturation at one residue and further improving it by using the improved variant as the template for site-saturation

at another residue [5]. ISM has proven to be successful for improving thermostability of *Bacillus subtilis* lipase A and enantioselectivity of an epoxide hydrolase from *Aspergillus niger* [5].

A subset of ISM that can also be employed is combinatorial active site saturation testing (CASTing). This method generates a library by randomization at several sets of two or three spatially close residue positions. The groups are chosen based on their secondary structure which allows for possible synergistic effects to be seen. The above stated example of improving the enantioselectivity of an epoxide hydrolase from *Aspergillus niger* utilized CASTing [6].

#### 5.2.1.2 Residues to Target for Further Improvements to Penicillin G Acylase Selectivity

In the work presented in this dissertation, we improved the selectivity of PGA by site-saturating residues within 6 Å of the alpha carbon ( $C_\alpha$ ) of the inhibitor penicillin G sulfoxide with NNK libraries. Since we have not exhausted the first and second shell residues in the active site of PGA, residues should continue to be chosen based on their proximity to the  $C_\alpha$  of penicillin G sulfoxide for ISM to further improve the selectivity of PGA. In addition to the 6 residues that have already been chosen, there are 11 additional residues that are within the 8 Å that should be targeted for mutation. Again, residues that are known to be necessary for catalysis should not be considered for mutation. The 11 residues are shown in Figure 5.1 and listed with their secondary structures in Table 5.1. Of the residues that have not already been investigated, only  $\alpha$ Thr141 and  $\alpha$ Arg145 could be combined to form a CAST library. Alternatively, the residues that have not been investigated can be combined with the residues that have been investigated to form the

following CASTing libraries: (1)  $\beta$ Phe24 and  $\beta$ Gly25; (2)  $\alpha$ Met142 and  $\alpha$ Phe146; and (3)  $\beta$ Gly70 and  $\beta$ Phe71. Since  $\beta$ Phe24 and  $\alpha$ Phe146 have proven to alter the selectivity of PGA in our studies, CASTing libraries 1 and 2 would be of particular interest.

Table5.1: Residues to target for further improvements to PGA Selectivity.

Residue	Secondary Structure
<i>Shell: 0 Å – 6 Å</i>	
$\alpha$ Phe146	Helix
$\beta$ Pro22	Loop
$\beta$ Phe24	Loop
$\beta$ Thr68	Sheet
$\beta$ Phe71	Loop
<i>Shell: 6 Å – 8 Å</i>	
$\alpha$ Thr141	Helix
$\alpha$ Met142	Helix
$\alpha$ Arg145	Helix
$\beta$ Asn2	Sheet
$\beta$ Gly21	Sheet
$\beta$ Gly25	Loop
$\beta$ Tyr31	Loop
$\beta$ Phe57	Sheet
$\beta$ Ser67	Sheet
$\beta$ Gly70	Loop
$\beta$ Ile177	Sheet

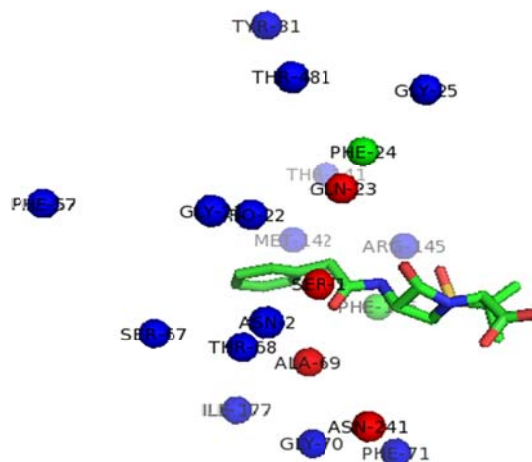


Figure 5.1: Crystal structure showing the active site of PGA complexed with penicillin G sulfoxide (PDB 1GM9). Penicillin G sulfoxide is shown in stick representation. Residues that were chosen for mutation are labeled and shown in ball representation. Residues that are known to be necessary for catalysis are shown in red in ball representation.

### 5.2.1.3 Potential Obstacles

We have experience in creating and screening diverse NNK libraries for single residues and therefore, we expect obstacles for site-saturation at one position to be minimal. However, if CASTing libraries were chosen for study, efforts should be taken to make screening more feasible. The current choice of utilizing a NNK restricted library that codes for 32 codons requires screening of at least 94 mutants for saturation at one position in order to guarantee that the libraries are covered with greater than 95% confidence. If two residues were chosen for simultaneous NNK site-saturation, 3066 mutants would need to be screened to cover the library space with greater than 95% confidence. In the present work we used an ampicillin synthesis-based HPLC assay that required 40 minutes screening per mutant. For 94 mutants, the assay is arduous, but feasible. For 3066 mutants, the current HPLC screen method would be extremely time-consuming. A possible solution would be to use a further restricted NDT codon library (N represents an equal mixture of the nucleotide bases A, G, C, and T; D represents an equal mixture of A, G, and T) when simultaneous saturation at two positions is desired. NDT libraries code for a diverse set of 12 amino acids instead of all 20 proteinogenic amino acids that NNK libraries code for. However, switching to a NDT library would reduce the number of condons to 12 and the number of mutants required to be screened for greater than 95% coverage to 430.

### **5.2.2 Decouple Reactions in Kinetically-Controlled Synthesis of Ampicillin with Racemic and Pure Substrates to Further Study Selective PGA Variants**

As discussed in Chapter 4 of this dissertation, we have found that the selective PGA variants that we have identified have racemase or epimerase activity. Such activity

has never been reported for PGA or any PGA variant. This phenomenon is fascinating, but cannot be explained with existing knowledge of PGA. At this point we are not even able to determine if the enzyme is acting as a racemase or an epimerase because our current analytical capabilities only allow us to distinguish between diastereomers. To begin to understand this phenomenon, chiral analytical techniques must be pursued and the reactions in the kinetically-controlled synthesis of ampicillin must be decoupled to paint a clearer picture of the system.

#### 5.2.2.1 Development of Chiral High Performance Liquid Chromatography Analysis

A number of Astec CHIROBIOTIC® T Chiral HPLC Columns that employ teicoplanin as a chiral selector to separate amino acids are readily available from Sigma Aldrich (St. Louis, MO, USA). The method must analyze for seven components – (*R*)-phenylglycine ((*R*)-PG), (*S*)-phenylglycine ((*S*)-PG), (*R*)-ampicillin, (*S*)-ampicillin, (*R*)-phenylglycine methyl ester, (*S*)-phenylglycine methyl ester, and 6-APA.

With a chiral HPLC method in hand, the reactions should be decoupled as follows: (1) hydrolysis of *rac*-PGME to form (*R*)-PG and (*S*)-PG; (2) hydrolysis of *rac*-ampicillin to form 6-APA, (*R*)-PG, and (*S*)-PG; and (3) synthesis of ampicillin from *rac*-PGME and 6-APA to form (*R*)-ampicillin and (*S*)-ampicillin. After the reactions are decoupled, it will be clear if the enzyme has racemase activity or epimerase activity or both.



#### 5.2.2.2 Potential Obstacles

(*S*)-ampicillin is not readily available, so it will need to be synthesized and purified. We are able to synthesize (*S*)-ampicillin enzymatically with the use of wild-type PGA as discussed in Chapter 4, albeit at low yields. The main obstacle that will be encountered is how to isolate (*S*)-ampicillin given its high solubility in the presence of phenylglycine. DSM (Delft, Netherlands) reports that the isolation of ampicillin is accomplished by acidification of the reaction mixture followed by a selective crystallization with a pH shift [7]. A potential solution would be to study amoxicillin as opposed to ampicillin. The variant  $\beta$ Phe24Ala has been previously reported to have a higher  $k_{cat}/K_m$  for (R)-p-hydroxyphenylglycine methyl ester, the side chain acyl donor for amoxicillin, than it does for (R)-PGME, the side chain acyl donor for ampicillin [8]. Therefore, such a switch should not be limited by substrate specificity. The isolation of amoxicillin is much easier than ampicillin because its solubility is much lower, and therefore the crystalline product can be removed from the reaction mixture by filtration [7].

### 5.3 References

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## **APPENDIX A**

### **THE UNITED STATES FOOD AND DRUG ADMINISTRATION APPROVED $\beta$ -LACTAM ANTIBIOTICS**

Table A.1: The United States Food and Drug Administration approved  $\beta$ -lactam antibiotics including their classifications, preparations and manufacturing companies. The antibiotics on the WHO's Model List of Essential Medicines are marked with a  $\checkmark$  if they are on the core list and a  $\dagger$  if they are on the complementary list.

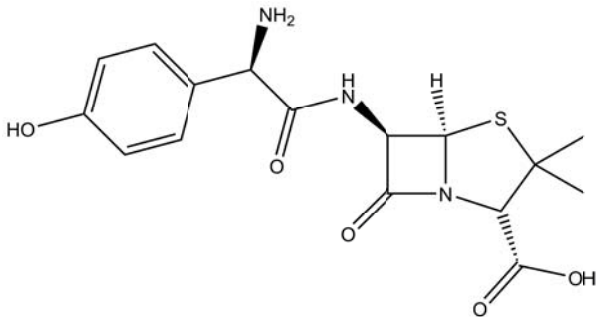
Classification	FDA Approved $\beta$ -Lactam Antibiotic	Preparations	Manufacturing Companies
penicillins	amoxicillin $\checkmark$	amoxicillin	AM Antibiotics; Apothecon; Aurobindo Pharma; Clonmel Healthcare; DAVA Pharmaceuticals, Inc.; GlaxoSmithKline; Hikma Pharmaceuticals PLC Pharms; MiddleBrook Pharmaceuticals, Inc.; Morton Grove Pharmaceuticals; Ranbaxy Laboratories Limited; Sandoz; Teva Pharmaceutical Industries, Ltd.
		amoxicillin; clavulanate potassium	Apotex Inc; GlaxoSmithKline; Hikma Pharmaceuticals PLC Pharms; Lek, a Sandoz Company; Morton Grove Pharmaceuticals; Ranbaxy Laboratories Limited; Sandoz; Smithkline Beechman Teva Pharmaceutical Industries, Ltd.
		amoxicillin; clarithromycin; lansoprazole	Takeda Pharmaceuticals North America, Inc.
penicillins	ampicillin $\checkmark$	ampicillin sodium	Hanford Pharmacueticals; Instituto Biochemico; Sandoz
		ampicillin/ampicillin trihydrate	Apothecon; Clonmel Healthcare; Purepac Pharmaceutical Co Pharm; Sandoz

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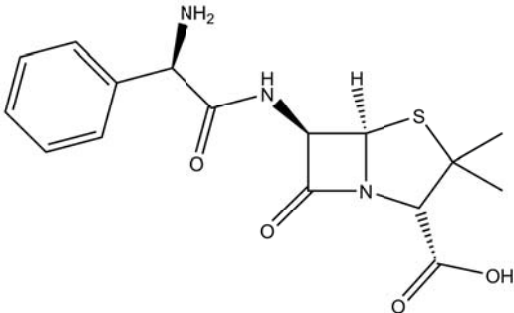
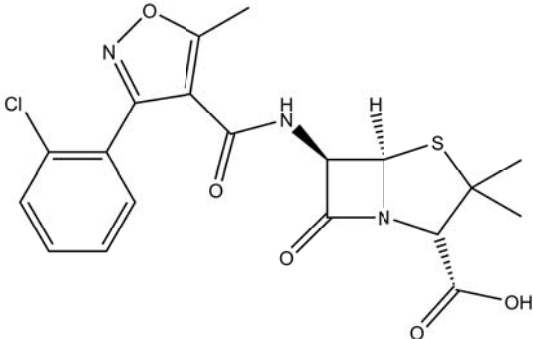
Classification	FDA Approved $\beta$ -Lactam Antibiotic	Preparations	Manufacturing Companies
		ampicillin sodium; sulbactam sodium	Baxter International Inc.; GeneraMedix Inc.; Hanford Pharmaceuticals; Istituto Biochimico; Pfizer Inc.; Sandoz
penicillins	cloxacillin $\checkmark$ 	cloxacillin sodium	GlaxoSmithKline

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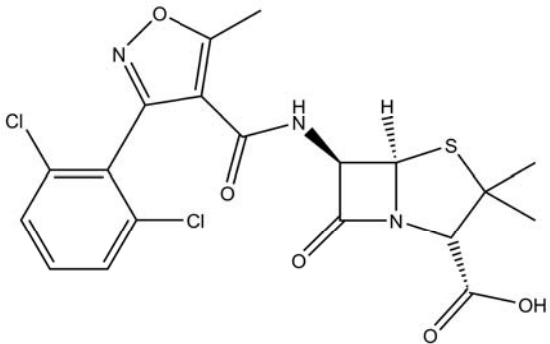
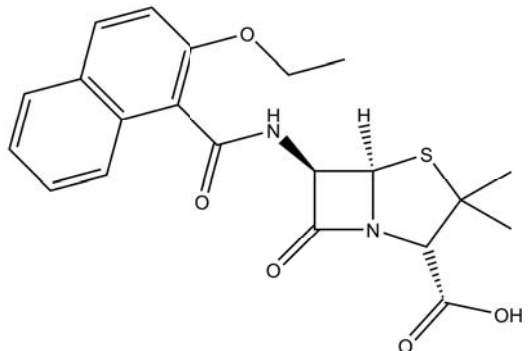
Classification	FDA Approved $\beta$ -Lactam Antibiotic	Preparations	Manufacturing Companies
penicillins	dicloxacillin 	dicloxacillin sodium	Sandoz ; Teva Pharmaceutical Industries, Ltd.
penicillins	nafcillin 	nafcillin sodium	Baxter International Inc.; Sandoz

Table A.1: The United States Food and Drug Administration approved  $\beta$ -lactam antibiotics including their classifications, preparations and manufacturing companies. The antibiotics on the WHO's Model List of Essential Medicines are marked with a  $\checkmark$  if they are on the core list and a  $\dagger$  if they are on the complementary list.

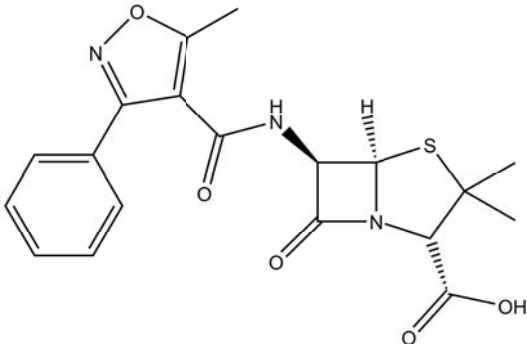
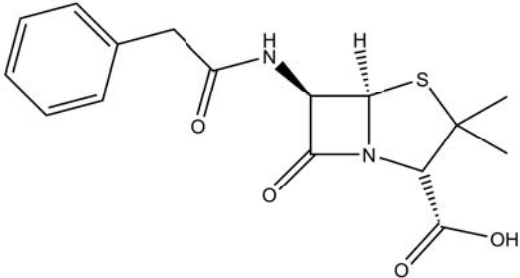
Classification	FDA Approved $\beta$ -Lactam Antibiotic	Preparations	Manufacturing Companies
penicillins	oxacillin 	oxacillin sodium	Baxter International Inc.; GlaxoSmithKline; Marsam Pharmaceuticals Inc.; Sandoz
penicillins	penicillin G $\checkmark$ 	penicillin G benzathine penicillin G benzathine; penicillin G procaine penicillin G potassium penicillin G procaine penicillin G sodium	King Pharmaceuticals, Inc.; Pfizer Inc. King Pharmaceuticals, Inc. Baxter International Inc.; Pfizer Inc.; Sandoz King Pharmaceuticals, Inc. Sandoz

Table A.1: The United States Food and Drug Administration approved  $\beta$ -lactam antibiotics including their classifications, preparations and manufacturing companies. The antibiotics on the WHO's Model List of Essential Medicines are marked with a  $\checkmark$  if they are on the core list and a  $\dagger$  if they are on the complementary list.

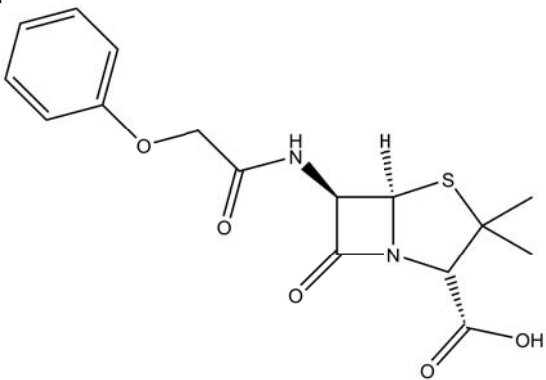
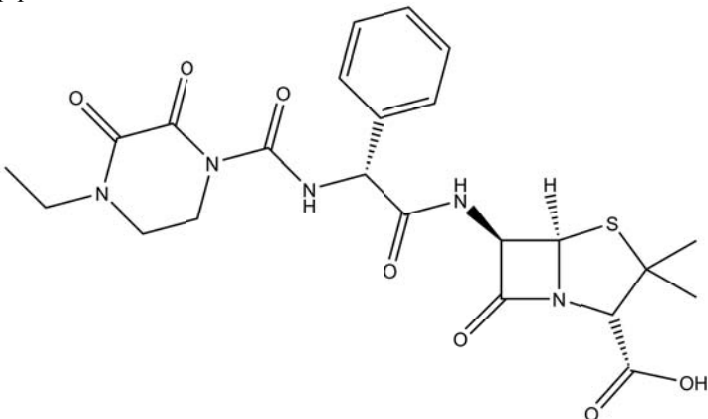
Classification	FDA Approved $\beta$ -Lactam Antibiotic	Preparations	Manufacturing Companies
penicillins	penicillin V $\checkmark$ 	penicillin V potassium	AM Antibiotics; Aurobindo Pharma; Clonmel Healthcare; Sandoz; Teva Pharmaceutical Industries, Ltd.
penicillins	piperacillin 	piperacillin sodium piperacillin sodium; tazobactam sodium	Istituto Biochimico Wyeth Pharmaceuticals



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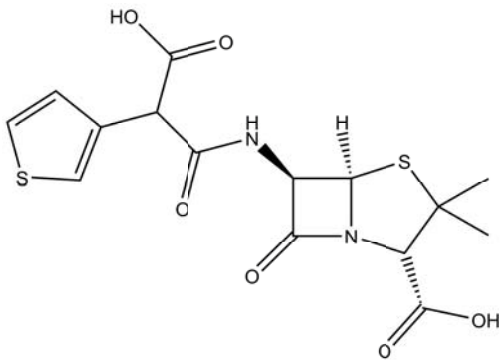
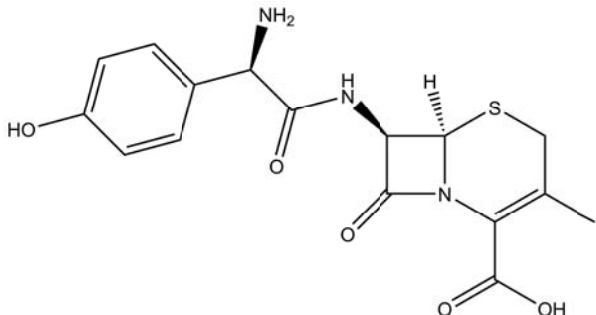
Classification	FDA Approved $\beta$ -Lactam Antibiotic	Preparations	Manufacturing Companies
penicillins	ticarcillin 	ticarcillin disodium; clavulanate potassium	GlaxoSmithKline
1 <sup>st</sup> generation cephalosporins	cefadroxil 	cefadroxil/cefadroxil hemihydrate	Aurobindo Pharma; Hikma Pharmaceuticals PLC; IVAX Pharmaceuticals, Inc.; Lupin Pharmaceuticals, Inc.; Orchid Chemicals & Pharmaceuticals Ltd.; Ranbaxy Laboratories Limited; Sandoz; Teva Pharmaceutical Industries, Ltd.; Warner Chilcott

Table A.1: The United States Food and Drug Administration approved  $\beta$ -lactam antibiotics including their classifications, preparations and manufacturing companies. The antibiotics on the WHO's Model List of Essential Medicines are marked with a  $\checkmark$  if they are on the core list and a  $\dagger$  if they are on the complementary list.

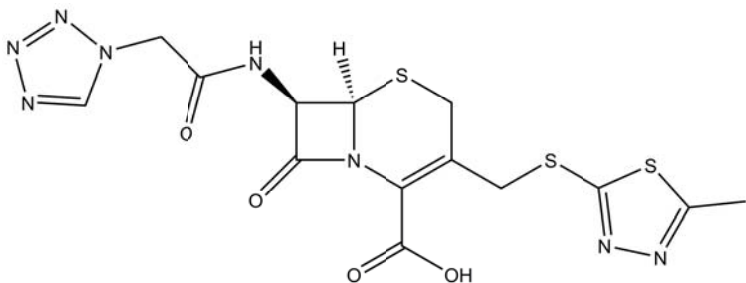
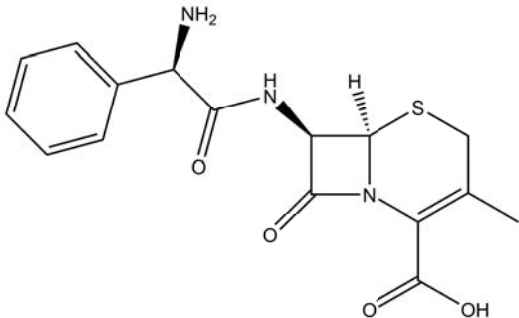
Classification	FDA Approved $\beta$ -Lactam Antibiotic	Preparations	Manufacturing Companies
1 <sup>st</sup> generation cephalosporins	cefazolin $\checkmark$ 	cefazolin sodium	APP Pharmaceuticals; ACS Dobfar Aurobindo Pharma' B. Braun Medical Inc.' Baxter International Inc.; Cephazone Pharma, LLC; GlaxoSmithKline; Hanford Pharmaceuticals; Hikma Pharmaceuticals PLC; Orchid Chemicals & Pharmaceuticals Ltd.; Samson Medical Technologies, L.L.C.; Sandoz
1 <sup>st</sup> generation cephalosporins	cephalixin 	cephalexin	Aurobindo Pharma; Belcher; Ceph International; Hikma Pharmaceuticals PLC; IVAX Pharmaceuticals, Inc.; Lex Pharmaceuticals Inc; Lupin Pharmaceuticals, Inc.; Orchid Chemicals & Pharmaceuticals Ltd.; Ranbaxy Laboratories Limited; Jerome Stevens Pharmaceuticals, Inc.; Sun Pharmaceutical Industries Ltd.; Teva Pharmaceutical Industries, Ltd.; Yung Shin Pharmaceutical

Table A.1: The United States Food and Drug Administration approved  $\beta$ -lactam antibiotics including their classifications, preparations and manufacturing companies. The antibiotics on the WHO's Model List of Essential Medicines are marked with a  $\checkmark$  if they are on the core list and a  $\dagger$  if they are on the complementary list.

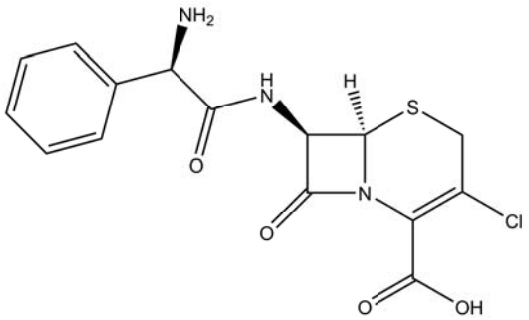
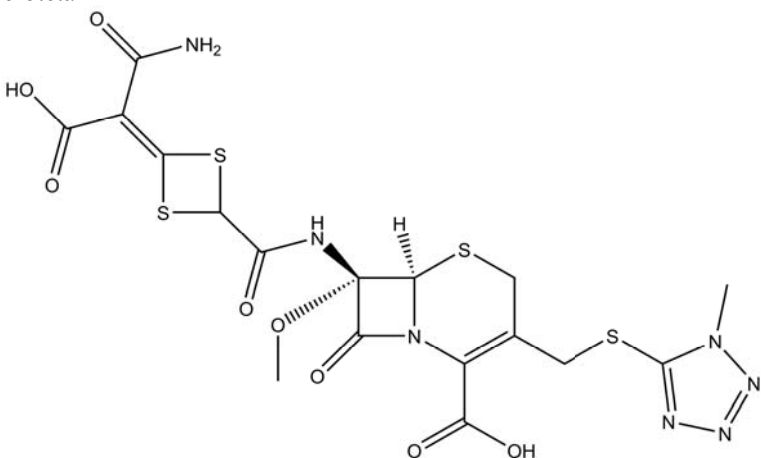
Classification	FDA Approved $\beta$ -Lactam Antibiotic	Preparations	Manufacturing Companies
2 <sup>nd</sup> generation cephalosporins	cefaclor 	cefaclor	Ceph International; Hikma Pharmaceuticals PLC; Marsam Pharmaceuticals Inc.; Par Pharmaceutical Companies, Inc.; Ranbaxy Laboratories Limited; Teva Pharmaceutical Industries, Ltd.; Yung Shin Pharmaceutical
2 <sup>nd</sup> generation cephalosporins	cefotetan 	cefotetan disodium	APP Pharmaceuticals; B. Braun Medical Inc.

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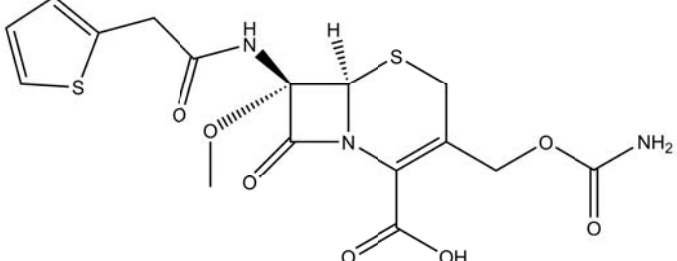
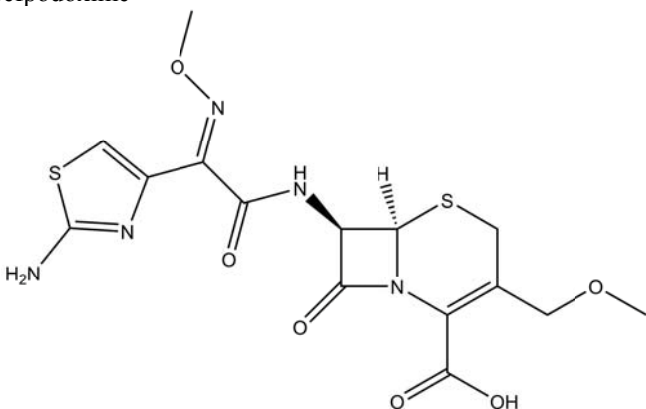
Classification	FDA Approved $\beta$ -Lactam Antibiotic	Preparations	Manufacturing Companies
2 <sup>nd</sup> generation cephalosporins	cefoxitin 	cefoxitin sodium	APP Pharmaceuticals; ACS Dobfar B. Braun Medical Inc.; Baxter International Inc.; Bioniche Pharma; Orchid Chemicals & Pharmaceuticals Ltd.
2 <sup>nd</sup> generation cephalosporins	cefpodoxime 	cefpodoxime proxetil	Aurobindo Pharma; Orchid Chemicals & Pharmaceuticals Ltd.; Pharmacia and Upjohn; Ranbaxy Laboratories Limited; Sandoz

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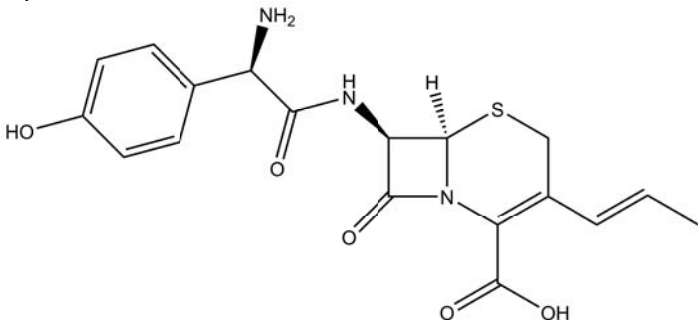
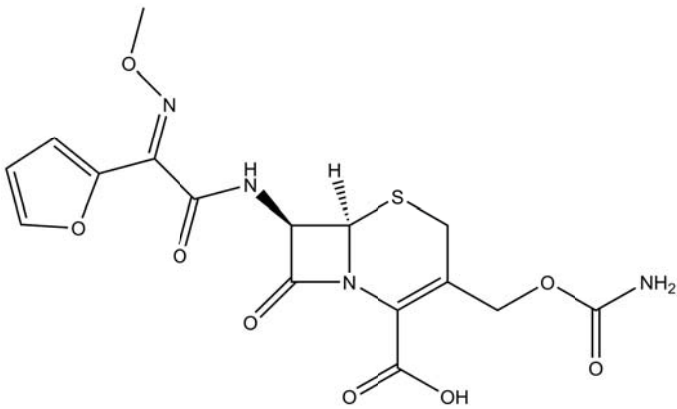
Classification	FDA Approved $\beta$ -Lactam Antibiotic	Preparations	Manufacturing Companies
2 <sup>nd</sup> generation cephalosporins	cefprozil 	cefprozil	Apotex Inc; Aurobindo Pharma; Bristol-Myers Squibb Company; Lupin Pharmaceuticals, Inc.; Orchid Chemicals & Pharmaceuticals Ltd.; Ranbaxy Laboratories Limited; Sandoz; Teva Pharmaceutical Industries, Ltd.; Wockhardt
2 <sup>nd</sup> generation cephalosporins	cefuroxime 	cefuroxime axetil  cefuroxime sodium	Apotex Inc; Aurobindo Pharma; GlaxoSmithKline; Lupin Pharmaceuticals, Inc.; Orchid Chemicals & Pharmaceuticals Ltd.; Ranbaxy Laboratories Limited; Sandoz; Teva Pharmaceutical Industries, Ltd.; Wockhardt  APP Pharmaceuticals; B. Braun Medical Inc.; GlaxoSmithKline; Hanford Pharmaceuticals; Hikma Pharmaceuticals PLC; Marsam Pharmaceuticals Inc.; Orchid Chemicals & Pharmaceuticals Ltd.; Teva Pharmaceutical Industries, Ltd.

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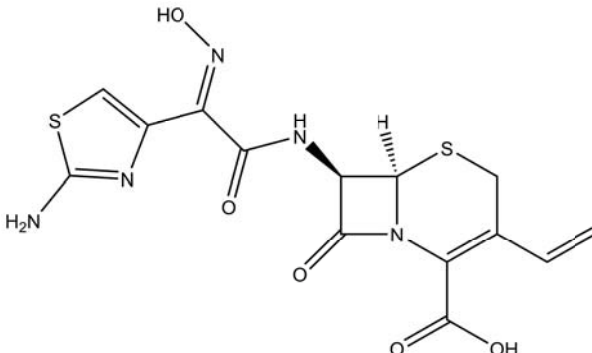
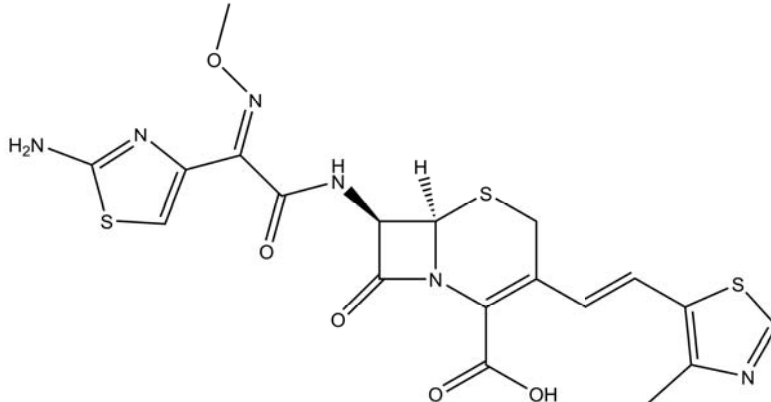
Classification	FDA Approved $\beta$ -Lactam Antibiotic	Preparations	Manufacturing Companies
3 <sup>rd</sup> generation cephalosporins	cefdinir 	cefdinir	Abbott Laboratories; Aurobindo Pharma Lupin Pharmaceuticals, Inc.; Orchid Chemicals & Pharmaceuticals Ltd.; Sandoz ; Teva Pharmaceutical Industries, Ltd.
3 <sup>rd</sup> generation cephalosporins	cefditoren 	cefditoren pivoxil	Spectracef Cornerstone

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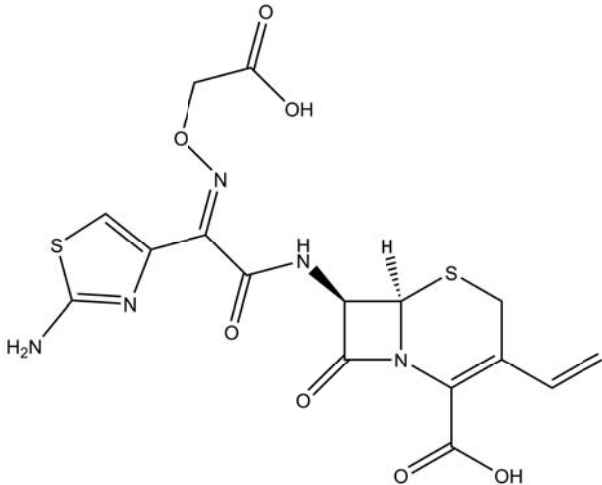
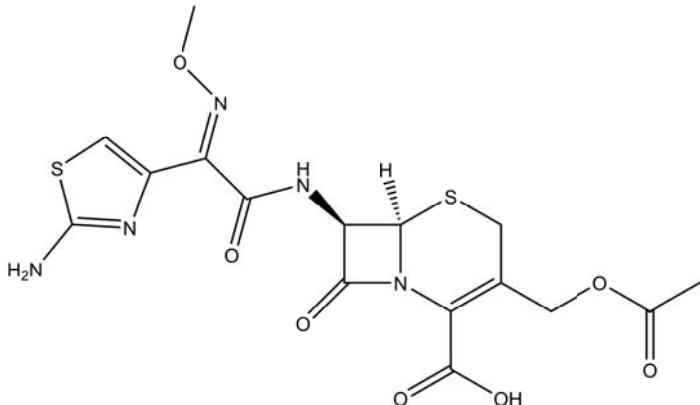
Classification	FDA Approved $\beta$ -Lactam Antibiotic	Preparations	Manufacturing Companies
3 <sup>rd</sup> generation cephalosporins	cefixime $\checkmark$ 	cefixime	Lupin Pharmaceuticals, Inc. Pharms
3 <sup>rd</sup> generation cephalosporins	cefotaxime 	cefotaxime sodium	APP Pharmaceuticals; Hikma Pharmaceuticals PLC; Lupin Pharmaceuticals, Inc.; Orchid Chemicals & Pharmaceuticals Ltd.; Sanofi-aventis U.S. LLC.; Wockhardt

Table A.1: The United States Food and Drug Administration approved  $\beta$ -lactam antibiotics including their classifications, preparations and manufacturing companies. The antibiotics on the WHO's Model List of Essential Medicines are marked with a  $\checkmark$  if they are on the core list and a  $\dagger$  if they are on the complementary list.

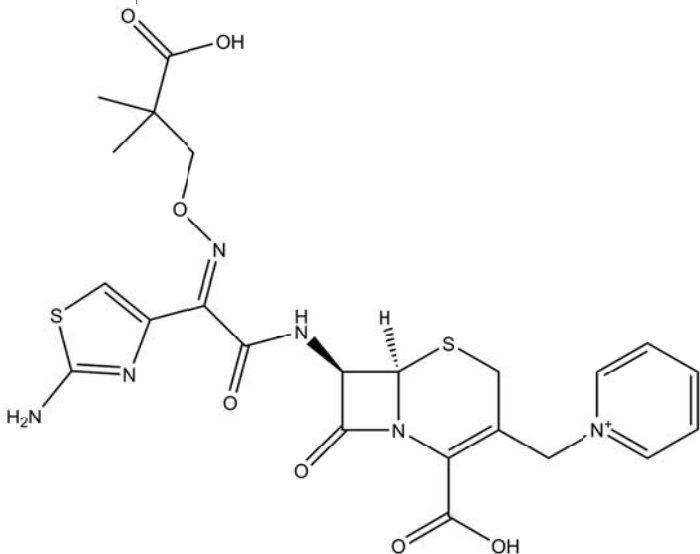
Classification	FDA Approved $\beta$ -Lactam Antibiotic	Preparations	Manufacturing Companies
3 <sup>rd</sup> generation cephalosporins	<div> <div>ceftazidime <math>\dagger</math></div>  </div>	ceftazidime	ACS Dobfar; GlaxoSmithKline; Hospira, Inc.; Wockhardt
		ceftazidime sodium	GlaxoSmithKline



Table A.1: The United States Food and Drug Administration approved  $\beta$ -lactam antibiotics including their classifications, preparations and manufacturing companies. The antibiotics on the WHO's Model List of Essential Medicines are marked with a  $\checkmark$  if they are on the core list and a  $\dagger$  if they are on the complementary list.

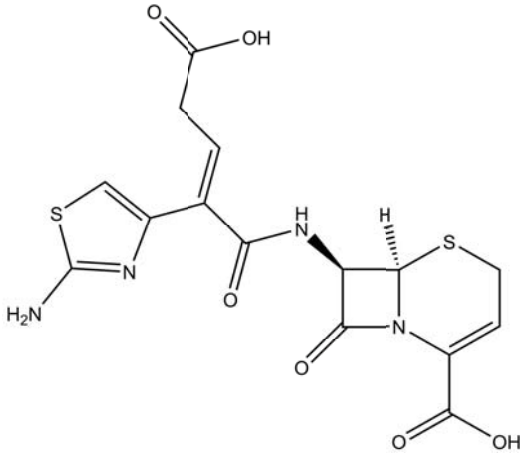
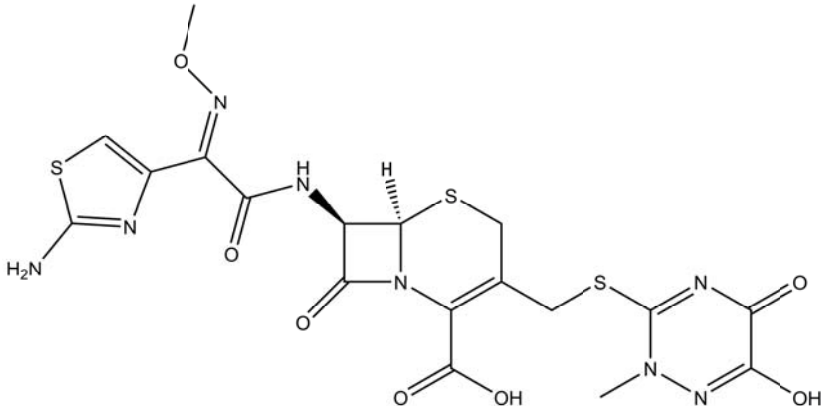
Classification	FDA Approved $\beta$ -Lactam Antibiotic	Preparations	Manufacturing Companies
3 <sup>rd</sup> generation cephalosporins	ceftibuten 	ceftibuten dehydrate	Sciele Pharma, Inc.
3 <sup>rd</sup> generation cephalosporins	ceftriaxone $\dagger$ 	ceftriaxone sodium	APP Pharmaceuticals; ACS Dobfar Aurobindo Pharma; B. Braun Medical Inc.; Baxter International Inc.; Bedford Laboratories; Cephalone Pharma, LLC; Hanford Pharmaceuticals; Hikma Pharmaceuticals PLC; HLR Luitpold Pharmaceuticals, Inc.; Lupin Pharmaceuticals, Inc.; Orchid Chemicals & Pharmaceuticals Ltd.; Sandoz; Teva Pharmaceutical Industries, Ltd.; Wockhardt

Table A.1: The United States Food and Drug Administration approved  $\beta$ -lactam antibiotics including their classifications, preparations and manufacturing companies. The antibiotics on the WHO's Model List of Essential Medicines are marked with a  $\checkmark$  if they are on the core list and a  $\dagger$  if they are on the complementary list.

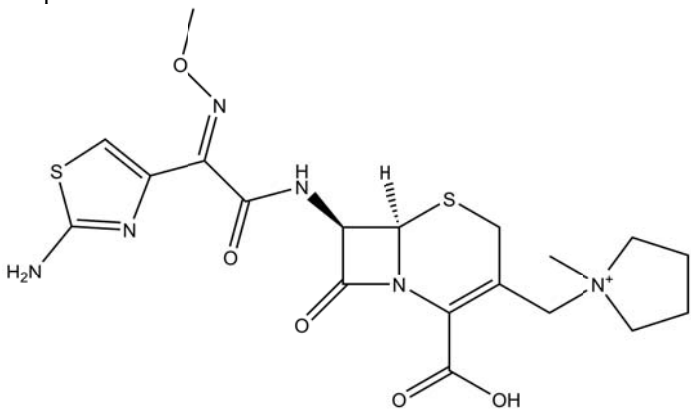
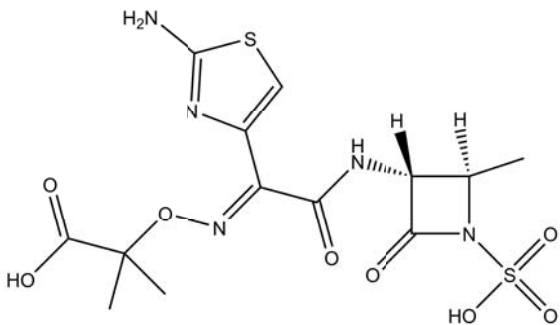
Classification	FDA Approved $\beta$ -Lactam Antibiotic	Preparations	Manufacturing Companies
4 <sup>th</sup> generation cephalosporins	cefepime 	cefepime hydrochloride	ACS Dobfar; Baxter International Inc.; Bristol-Myers Squibb Company; Orchid Chemicals & Pharmaceuticals Ltd.
monobactams	aztreonam 	aztreonam	Bristol-Myers Squibb Company

Table A.1: The United States Food and Drug Administration approved  $\beta$ -lactam antibiotics including their classifications, preparations and manufacturing companies. The antibiotics on the WHO's Model List of Essential Medicines are marked with a  $\checkmark$  if they are on the core list and a  $\dagger$  if they are on the complementary list.

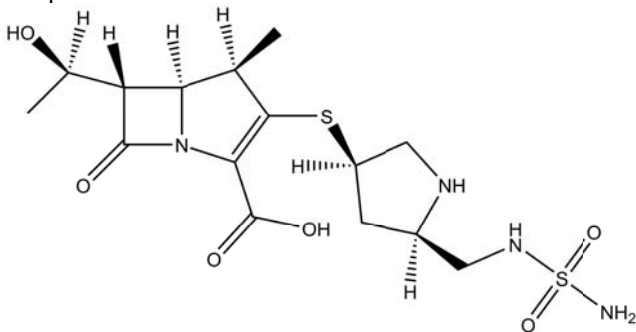
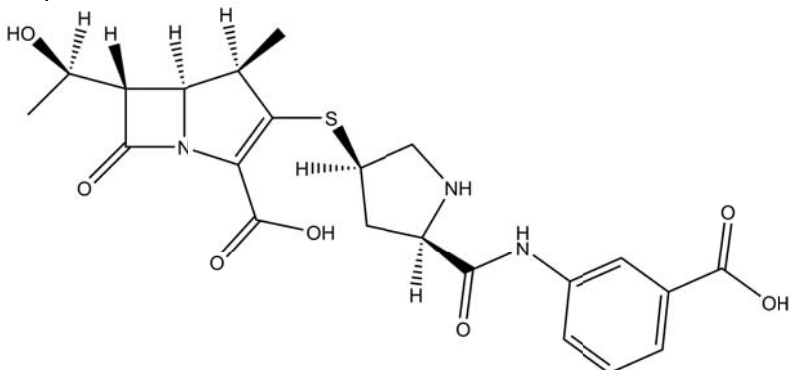
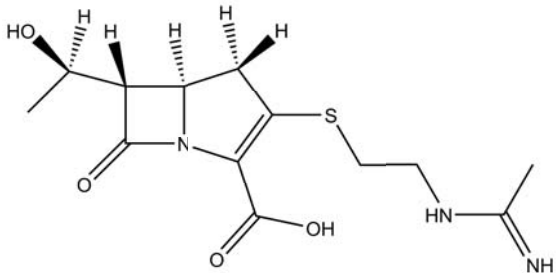
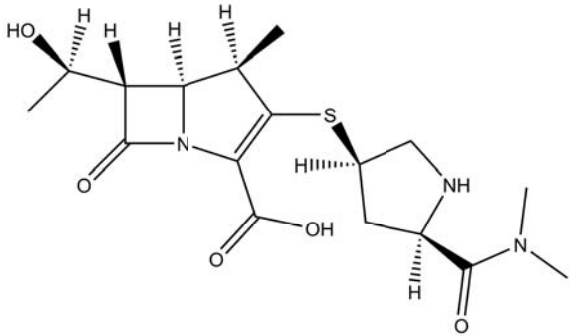
Classification	FDA Approved $\beta$ -Lactam Antibiotic	Preparations	Manufacturing Companies
carbapenems	<p>Doripenem</p> 	doripenem	Johnson & Johnson
carbapenems	<p>Ertapenem</p> 	ertapenem sodium	Merck & Co., Inc.

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Classification	FDA Approved $\beta$ -Lactam Antibiotic	Preparations	Manufacturing Companies
carbapenems	imipenem $\dagger$ 	imipenem; cilastatin sodium	Merck & Co., Inc.
carbapenems	Meropenem 	Meropenem	AstraZeneca International

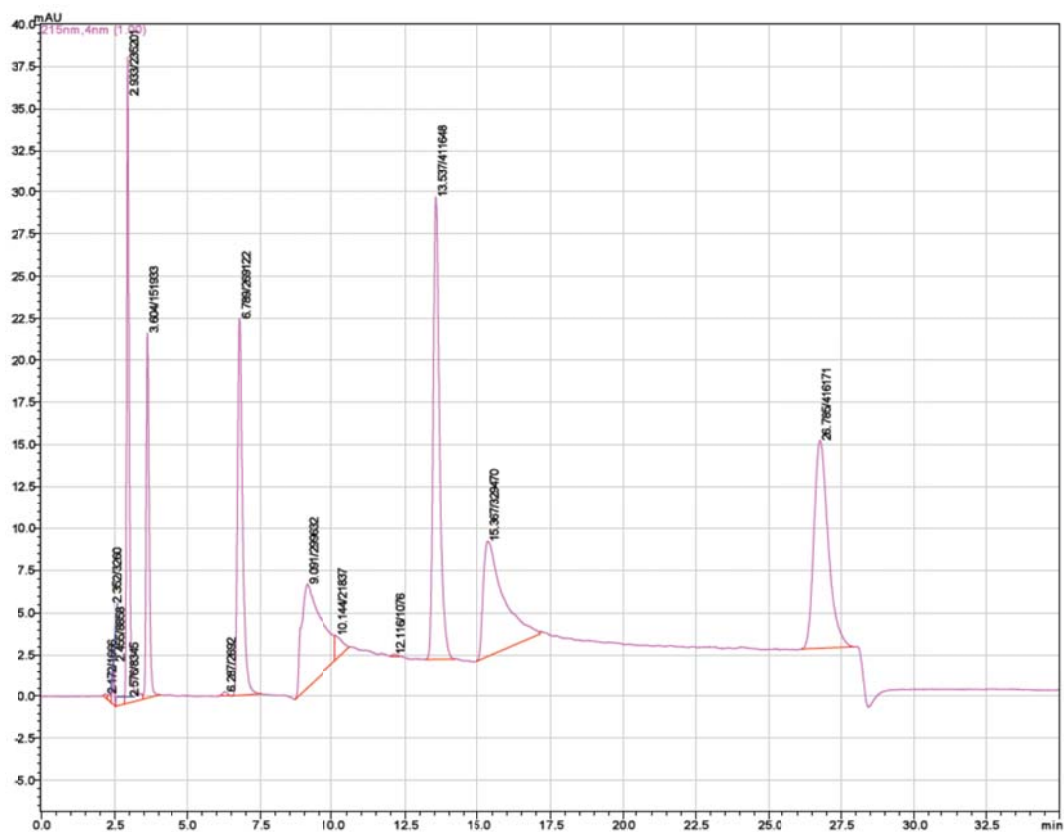
## **APPENDIX B**

### **ANATLYTICAL METHODS**

HPLC Method:

Shimadzu-LC-20AT pump, Beckman Coulter Ultrasphere ODS 4.6 mm x 25 cm column, and SPD-M20A prominence diode array detector monitored at 215 nm.

Mobile phase operated under a gradient at 1.0 mL/min and contains 20% methanol and 80% 0.02 phosphate buffer at pH 7 and increased to 50% methanol over a 25 min period.



(*R*)-phenylglycine / RT = 2.93

6-aminopenicillanic acid / RT = 3.60

phenylacetic acid / RT = 6.79

(*R*)-ampicillin / RT = 13.54

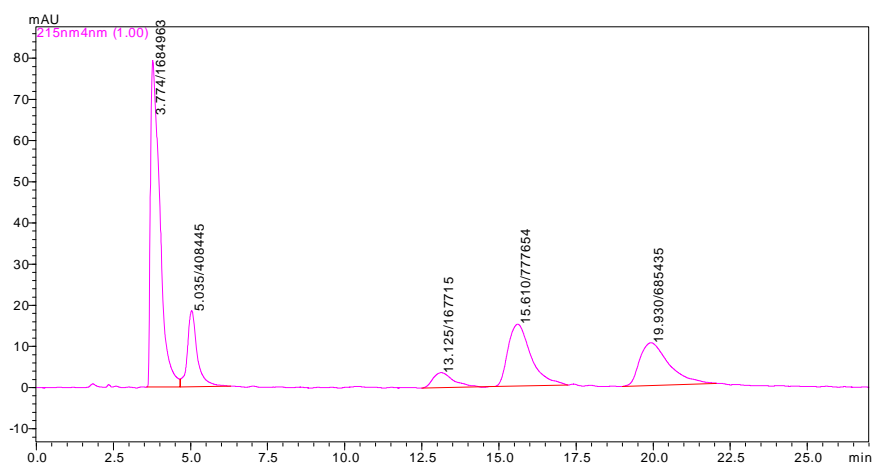
(*R*)-phenylglycine methyl ester / RT = 15.37

penicillin G / RT = 26.79

## HPLC Method:

Shimadzu-LC-20AT pump, Beckman Coulter Ultrasphere ODS 4.6 mm x 25 cm column, and SPD-M20A prominence diode array detector monitored at 215 nm.

Mobile phase was isocratic at  $1.0 \text{ mL min}^{-1}$  with 30% acetonitrile and 70% 5 mM phosphate buffer containing  $300 \text{ mg L}^{-1}$  sodium dodecyl sulfate at pH 2.65.



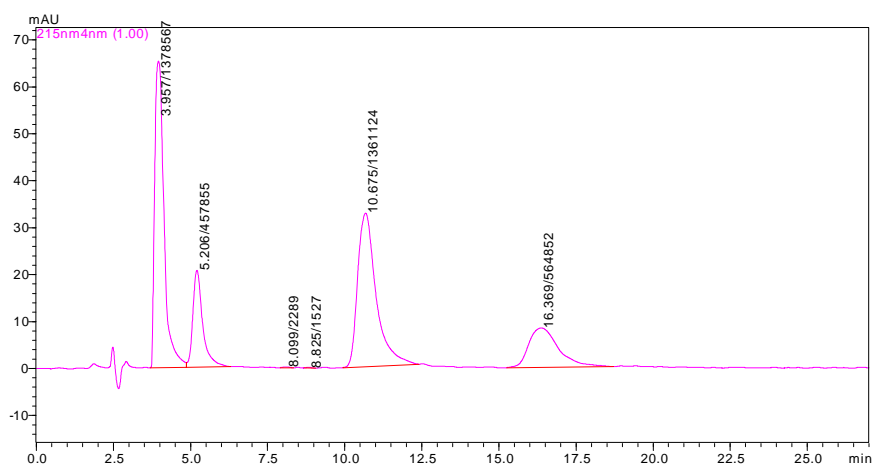
(*R*)-phenylglycine / RT = 3.77

6-aminopenicillanic acid / RT = 5.04

(*R*)-phenylglycine hydroxyethyl ester / RT = 13.13

(*R*)-ampicillin / RT = 15.61

(*R*)-phenylglycine methyl ester / RT = 19.93



(*R*)-phenylglycine / RT = 3.96

6-aminopenicillanic acid / RT = 5.20

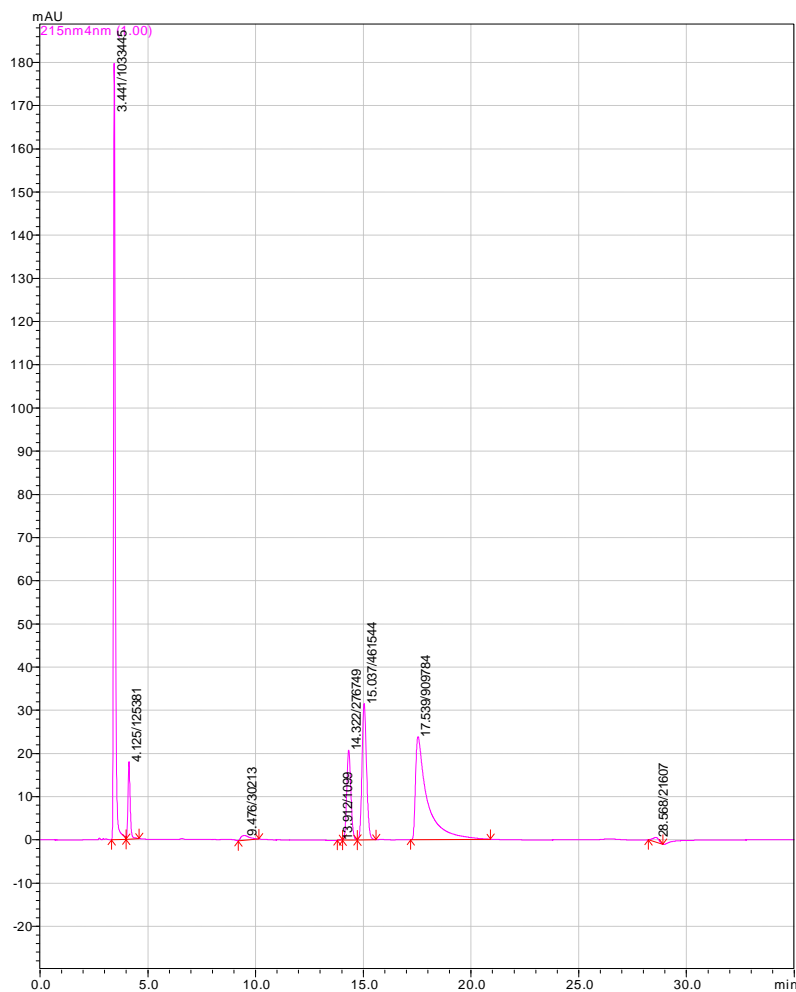
(*R*)-phenylglycine amide / RT = 10.68

(*R*)-ampicillin / RT = 16.37

### HPLC Method:

Shimadzu-LC-20AT pump, Beckman Coulter Ultrasphere ODS 4.6 mm x 25 cm column, and SPD-M20A prominence diode array detector monitored at 215 nm.

Mobile phase was isocratic at 1.0 mL min<sup>-1</sup> that initially contained 12.5% methanol and 87.5% 20 mM phosphate buffer at pH 7.0, increased via a step change to 30.0% methanol and 70% 20 mM phosphate buffer at pH 7.0 after 7 minutes, and decreased via a step change to 12.5% methanol and 87.5% 20 mM phosphate buffer at pH 7.0 after 30 min until 40 min.



*rac*-phenylglycine / RT = 3.44

6-aminopenicillanic acid / RT = 4.13

(*S*)-ampicillin / RT = 14.32

(*R*)-ampicillin / RT = 15.04

*rac*-phenylglycine methyl ester / RT = 17.54



## 2-Nitro-5-[(Phenylacetyl)amino]-Benzoic Acid Assay:

The hydrolysis of NIPAB liberates 5-amino-2-nitrobenzoic acid, which was monitored at 405 nm with a Beckmann-Coulter DU®800 spectrophotometer. NIPAB hydrolysis reactions were carried out at 25°C in 50 mM phosphate buffer at pH 7.5

