Design, Synthesis and Evaluation of Specific Substrates for *E. coli* Penicillin-Binding Protein 2

By

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Abstract

Each bacterial species generally expresses several enzymes that are responsible for catalyzing the final step of cell wall synthesis. All members of this family of enzymes are susceptible to inhibition by β-lactam antibiotics, and are consequently referred to as penicillin-binding proteins (PBPs). Unique amongst this family of enzymes in *E. coli* is PBP2, which is the only enzyme that is susceptible to inhibition by the amidinopenicillin mecillinam. Intriguingly, mecillinam is also unique amongst β-lactams, bearing an unusual, positively charged amidine side chain. Despite attracting significant interest over several decades, the nature of mecillinam’s exquisitely monogamous relationship with PBP2 remains obscure.

This lab has previously shown that a given peptidoglycan-mimetic side chain confers a similar change in target reactivity to both β-lactams and peptide substrates. This result would seem to imply that the mecillinam side chain, which dramatically increases the affinity and specificity of penicillin for *E. coli* PBP2, would therefore also increase the affinity and specificity of peptide and thiodepsipeptide substrates for PBP2. This thesis describes the creation of such compounds and their kinetic evaluation with *E. coli* PBP2. The results of these experiments suggest that, unlike the effect of the peptidoglycan-mimetic side chains, the mecillinam side chain confers increased PBP2 affinity only to penicillin and not to substrates. It remains unclear why *E. coli* PBP2 is able to bind and react with mecillinam, but not with substrates bearing the mecillinam side chain.
Sequence alignments of a variety of PBPs reveal that E. coli PBP2 contains an unusual SXD motif in its active site, rather than the SXN motif which is strictly conserved in most other PBPs. It is an intriguing coincidence, indeed, that E. coli PBP2 is unique in both its active site structure and in its reactivity with β-lactams. Astonishingly, this coincidence appears to have completely escaped discussion until now. This thesis explores each manifestation of the functional importance of the Asn-position of the SXN motif, including its role in substrate recognition, the reactivity of mecillinam with β-lactamases, the kinetic properties of a variety of SXN* mutants, the identification of bacterial species with SXD-containing PBPs, and molecular dynamics simulations of a homology model of E. coli PBP2.

The information compiled and examined in this thesis supports the hypothesis that Asp 132 of the SXD motif of E. coli PBP2 is solely responsible for the enzyme’s unusual behavior, and that an electrostatic interaction between the positively charged amidine and the negatively charged Asp is the source of mecillinam’s specificity for E. coli PBP2. This proposition suggests promising new directions for further characterization of this elusive enzyme.
I. INTRODUCTION
The Bacterial Cell Wall

Nearly all species of bacteria synthesize and maintain a cell wall, which acts as a rigid barrier between the cell and the environment. Bacteria are broadly divided into two classes based on their susceptibility to Gram stain. Gram-positive bacteria have a thick cell wall and lack an outer membrane, while Gram-negative bacteria have two cell membranes, with a relatively thinner cell wall layer between them (Figure 1.1). In both classes of bacteria, the cell wall is responsible for maintaining bacterial cell shape and protecting the cell from the effects of osmotic pressure.

Figure 1.1 – Comparison of the cell wall in Gram-positive and -negative bacteria.

From Voet, Voet and Pratt, 2002.¹

The cell wall is a three-dimensional polymer consisting of polysaccharide and short oligopeptides, and is therefore often referred to as peptidoglycan. The glycan strands are long, linear chains consisting of alternating amino sugars N-acetyl-glucosamine (NAG) and N-acetylmuramic acid (NAM) which are connected by β-1,4-glycosidic bonds. The lactyl group of the muramic acid residue is the site of
covalent attachment of the N-termini of the peptides which form the bridges that cross-link adjacent peptidoglycan strands (Figure 1.2). While the glycan strands vary little between bacterial species, the peptide cross bridges vary considerably. A common stem peptide consists of L-alanyl-D-γ-glutamyl-L-lysyl-D-alanyl-D-alanine, as in *Streptococcus sp.*, but the D-γ-glutamyl- and L-lysyl- positions vary between species. In *E. coli*, for example, the peptide is L-alanyl-D-glutamyl-*meso*-amino-pimelyl-D-alanyl-D-alanine (Figure 1.3). These strands of peptidoglycan are covalently bound to one another via the bridging peptides and, thus, the bacterial cell wall is one large, continuous polymer.

Figure 1.2 – Diagram of the three-dimensional structure of the cell wall peptidoglycan of *Staphylococcus aureus*. From Voet, Voet and Pratt, 2002.¹
The Penicillin-Binding Proteins

The final step in bacterial cell wall biosynthesis is the transpeptidation reaction, which forms the covalent bonds that cross-link adjacent strands of peptidoglycan. These reactions are catalyzed by a family of enzymes called the penicillin-binding proteins (PBPs), which are also called transpeptidases, D-alanyl-D-alanine peptidases, or DD-peptidases. These enzymes catalyze the nucleophilic attack of the amino group of the third residue in the pentapeptide of the acyl acceptor chain on the penultimate D-alanine of the acyl donor peptide chain (Figure 1.4).
Figure 1.4 – Schematic of the *Streptomyces* *sp.* peptidoglycan cross-linking reaction catalyzed by penicillin-binding proteins. From McDonough *et. al.*, 2002.²
The basic mechanism of the cross-linking reaction is well known and is shown in Scheme 1.1. To initiate the reaction, the PBP active site serine hydroxyl attacks the carbonyl carbon of the penultimate D-alanine of the acyl donor peptide, forming a tetrahedral intermediate, which quickly collapses to the more stable acyl-enzyme intermediate, with the terminal D-alanine serving as the leaving group. The acyl-enzyme intermediate can either be attacked by the free amine of the third residue of an acceptor stem peptide (transpeptidation) or by a water molecule (carboxypeptidation) to release the free enzyme. The product of the transpeptidation reaction is a new cross-link in the bacterial cell wall, while the carboxypeptidation reaction serves to trim the terminal D-alanine.3

As might be expected for a family of enzymes that catalyze the same reaction, the PBPs are all evolutionarily related, and it is useful to examine how each is different. The various PBPs have been broadly classified based on their size and amino acid sequence. The high-molecular weight (HMW) PBPs range from 100 to 60 kD and the low-molecular weight (LMW) PBPs range from 60 to 25 kD. Both the high- and low-molecular weight PBPs are divided into three classes, A, B and C, and each class contains subclasses based amino acid sequence, as determined by Ghuysen.3-5 The HMW PBPs of class A are the only PBPs that contain both transglycosylation and transpeptidation domains. The transglycosylase domain is responsible for the lengthening of the linear glycan chain from lipid II precursor molecules. Class B HMW PBPs contain a transpeptidase domain and another domain, lacking transglycosylase activity,6 whose function is still unclear but may be involved
in protein-protein recognition. All LMW PBPs contain only the transpeptidase domain. All HMW PBPs are anchored in the cell membrane by a string of hydrophobic amino acid residues at the N-terminal end of the protein, while many LMW PBPs are not membrane-bound.

Scheme 1.1 – Mechanism of the transpeptidation and carboxypeptidation reactions catalyzed by the penicillin-binding proteins.
Each bacterial species expresses several PBPs to carry out the various tasks of cell wall synthesis, maintenance and cell division. The array of PBPs in each species is usually a selection of a few enzymes of each class, and within the cell, each PBP is expressed in varying amounts. For example, *E. coli* contains two HMW class A enzymes, PBPs 1A and 1B, totalling a few hundred copies per cell, which are responsible for cell elongation and catalyze both the transpeptidation and the transglycosylation reactions. Other PBPs perform the transpeptidation reaction alone, but different PBPs seem to perform different functions with the same transpeptidation reaction. For example, the HMW Class B transpeptidase *E. coli* PBP2 is involved in cell growth and the maintenance of cell shape, while the transpeptidase *E. coli* PBP3 of the same class is involved in cell division and septum formation. Both *E. coli* PBP2 and PBP3 are expressed at only a few tens of copies per cell. The LMW PBPs, *E. coli* PBP5 for example, are expressed in the thousands of copies per cell, and appear to catalyze only the carboxypeptidation reaction, which limits the extent of cell wall cross-linking performed by the HMW PBPs.

In addition to catalyzing the formation of cell wall cross-bridges, the PBPs also bind and react with β-lactam antibiotics. Indeed, they were first discovered, and termed penicillin-binding proteins because of this phenomenon. When [¹⁴C]-labeled penicillins were incubated with *E. coli*, the labeled drug localized to proteins associated with the inner cell membrane. These labeled proteins could then be visualized when purified membranes were separated by gel electrophoresis (Figure 1.5). Several proteins were labeled with the isotope, and these previously undescribed
proteins were therefore named penicillin-binding proteins (PBPs). They were assigned numbers according to their descending molecular weight. The PBPs were later discovered to be the enzymes responsible for the transpeptidation reaction, as discussed above.

Penicillin is capable of reacting with the PBPs by nature of its structural similarity to the terminal D-alanyl-D-alanine of the peptidoglycan stem peptide, the natural substrate of the PBPs. Years before the targets of penicillin were identified, Tipper and Strominger noticed this structural similarity and postulated that penicillin was a potent inhibitor of bacterial cell wall synthesis.\(^8\) This hypothesis has been demonstrated to be true, and penicillin does in fact, react with PBPs in exactly the same way peptidoglycan substrates do, as is shown below in Scheme 1.2. The inhibitory nature of penicillin derives from the fact that, unlike the terminal D-alanine of the \textit{in vivo} peptide substrate, the five-membered ring of penicillin cannot leave, and this moiety physically blocks the entry of incoming acceptor peptide and water molecules. Thus, the penicillin acyl-enzyme intermediate is long lived, and the regeneration of the free enzyme is slow. When penicillin inactivates the PBPs in this way, the transpeptidation reaction cannot occur, no new cell wall cross-links can be formed, and the bacteria eventually lyse.
Figure 1.5 – Proteins from the inner membrane of *E. coli* are labeled by benzyl[^14C]penicillin. From Spratt, 1977.
Scheme 1.2 – Comparison of the reaction of PBPs with penicillin (top) and the terminal D-alanyl-D-alanine of the *in vivo* peptide substrate (bottom).
Mecillinam and *E. coli* PBP2

In 1972, Leo Pharmeceutical Company of Denmark reported the discovery of a new class of penicillins bearing an unusual amidine side chain, termed 6β-amidinopenicillanic acids. They displayed remarkable efficacy in killing Gram-negative bacteria, and had effects curiously different from those of the more common benzylpenicillin and ampicillin. The most effective of these novel antibiotics was termed mecillinam. The original term for mecillinam was FL1060, and it is sometimes referred to as simply amidinopenicillin or amdinocillin. Treatment of *E. coli* with mecillinam resulted in conversion of the rod-shaped cells to ovoid-shaped cells and eventual cell lysis.

Shortly thereafter, experiments using $[^{14}\text{C}]$-labeled mecillinam showed that mecillinam binds and reacts exclusively with *E. coli* PBP2, completely failing to label any other PBP (Figure 1.6). These experiments represented the first time a single PBP could be targeted and inactivated without concurrently affecting any other PBP. This unique behavior allowed for the effects of mecillinam to be assigned to PBP2 alone, and the resulting conclusion was that PBP2 is involved in the maintenance of
cell shape. This was the first identification of a PBP with an essential and defined role in bacterial cell growth. After this discovery, the role of each individual PBP in the synthesis of bacterial cell wall was explored by isolating bacterial strains with mutations in a single PBP gene.

Figure 1.6 – Mecillinam binds only to PBP2. From Spratt, 1975.
While mecillinam binds and reacts with PBP2 with remarkable affinity (more than an order of magnitude greater than benzylpenicillin, for example), many conventional β-lactams bind only poorly to PBP2. Indeed, some cephalosporins fail to bind to PBP2 whatsoever\(^7\) (Figure 1.7). This unusual behavior eventually allowed for the isolation and characterization of the reaction catalyzed by PBP2. In 1986, Ishino \textit{et al.} grew \textit{E. coli} which greatly overexpressed PBP2 (and RodA, an integral membrane protein which they demonstrated to be required for PBP2 activity) and treated the cells with cefoxitin, so that only PBP2 would remain catalytically active.\(^{12}\)

A transpeptidation reaction product was observed, and the reaction was mecillinam-sensitive, indicating the reaction was indeed catalyzed by PBP2.\(^{12}\) The reaction is shown below as Figure 1.8.
Figure 1.8 – The transpeptidation reaction catalyzed by *E. coli* PBP2.
Adapted from Ishino *et. al.*, 1986.12
The amino acid sequence of *E. coli* PBP2 was also determined in 1986\(^{13}\) and compared to the sequence of other PBPs. Alignments of all the amino acid sequences of penicillin-interacting proteins available at the time showed that several active site motifs are conserved in PBPs and β-lactamases across all bacterial species, including most importantly the reactive serine residue, followed by a lysine residue three positions later (S*XXK)\(^3\) (Figure 1.9). *E. coli* PBP2 appears to have a mutation in one of the conserved active site motifs. This mutation is unique amongst all other commonly examined PBPs, and occurs in the active site SXN motif which is conserved in PBPs across all bacterial species. The highly unusual SXD motif of *E. coli* PBP2 has been reported in at least three sequence alignments of Ghuysen,\(^{3-5}\) but has completely escaped notice and discussion in each report. Apparently, this thesis is the first work in which this mutation is discussed. It is interesting to consider the coincidence that *E. coli* PBP2 contains a unique SXD active site motif, and that it also is very unique in its reactivity with antibiotics.

Despite the fact that *E. coli* PBP2 holds an important place in the history of PBPs, it has largely fallen out of the spotlight since it garnered such intense interest in the 1970s. This may be due to several factors. Firstly, there is no crystal structure of PBP2, which limits structural studies on the enzyme. Secondly, there are no *in vitro* substrates of PBP2, which makes *in vitro* study of PBP2 activity difficult. In retrospect, this is not surprising, as in general, HMW PBPs, which represent the most critical β-lactam killing targets, fail to catalyze the turnover of most substrates *in*
vitrō.14, 15 These problems represent the largest barriers to understanding the function of *E. coli* PBP2 at the molecular level.

**Figure 1.9** – Sequence alignment of conserved active-site motifs of various PBPs.

The mutation in *E. coli* PBP2 is circled in red. Adapted from Ghuysen, 1991.3
Potential Specific Substrates of *E. coli* PBP2

*E. coli* PBP2 is a class B2 HMW PBP.\(^4\) It has an apparent molecular weight of 66,000, and is expressed in very small amounts, at approximately ten to twenty copies per cell.\(^7\),\(^11\) PBP2 is responsible for maintaining cell shape and, despite its low level of expression, PBP2 is necessary for cell survival. *In vivo*, it is associated with the integral membrane protein RodA, and this interaction is required for PBP2 activity *in vivo*,\(^12\) although not for its reactivity with β-lactams.\(^15\) A comparison of several PBPs’ reactivity with a variety of antibiotics (Figure 1.7) shows that *E. coli* PBP2 is unique in its behavior, most importantly in that it is the only PBP which binds and reacts with mecillinam. Mecillinam is exquisitely specific for *E. coli* PBP2 and it is the only penicillin which bears an unusual amidine linker to the side chain rather than the usual amide linker and thus, it is presumably the source of mecillinam’s specificity for *E. coli* PBP2.

Previous work in this lab has shown that peptidoglycan-mimetic side chains had the same effect (positive or negative) on both peptide substrate and β-lactam reactivity. Peptidoglycan-mimetic side chains greatly increased the affinity of peptide substrates and β-lactams for the R61 DD-peptidase, for example, but failed to increase affinity of substrates or β-lactams for *E. coli* PBP2.\(^15\) It is known, however, that mecillinam reacts rapidly with *E. coli* PBP2, and a tantalizing possibility is that the amidine side chain that so greatly increases penicillin affinity for PBP2 would also increase the affinity of peptide substrates for *E. coli* PBP2. Currently, there are no known substrates that react with *E. coli* PBP2 *in vitro.*
This thesis project attempted to create the first \textit{in vitro} substrates of \textit{E. coli} PBP2. Based on the exquisite specificity of mecillinam for \textit{E. coli} PBP2, we decided to use the mecillinam side chain with the amidine linker, and replace the penicilloyl moiety with D-alanyl-D-alanine to create the substrate 1. Thiodepsipeptide analogs of the dipeptide substrate, 2 and 3, were also synthesized, with the hope that they, too, would be substrates. If the substrates behave in a similar fashion to mecillinam, they might furthermore be expected to react specifically with \textit{E. coli} PBP2. This thesis also sheds light on the source of the unusual specificity observed in the reactivity of PBP2 with antibiotics.
II. SYNTHESIS AND CHARACTERIZATION OF 1
2.1 Materials

All $^1$H-NMR spectra were obtained from a Varian Mercury Vx 300 MHz spectrometer. Electrospray mass spectra were obtained from the Mass Spectrometry Laboratory at Univeristy of Illinois, Urbana-Champlain, School of Chemical Sciences, and from a Thermo-Fisher LTQ Advantage LC Mass Spectrometer. All size-exclusion column chromatography fractions were analyzed using a Hitachi U-2000 spectrophotometer. All HPLC purification was done with a Varian Prostar machine (Model 210 Solvent Delivery Modules and Model 340 UV-Vis Detector) with a Macherey-Nagel Nucleosil Column (C$_{18}$, 7 μm particle size, 300 Å pore size, octadecyl phase, end-capped, 10 mm ID, 250 mm length) and a Varian MetaGuard Nucleosil guard column (C$_{18}$, 5 μm particle size, 100 Å pore size, 4.6 mm length). A Mel-temp melting point apparatus was used to take melting points in open capillary tubes.

All $^2$H$_2$O (D$_2$O) and CDCl$_3$ were purchased from Cambridge Isotope Laboratories. Methanol, acetonitrile, and diethyl ether were purchased from Pharmaco-Aaper. Isopropanol, diisopropyl ether, and mercaptoacetic acid were purchased from Acros Organics. Benzene and formic acid were purchased from Spectrum Chemical Manufacturing Corporation. Triethylamine was purchased from Fluka Chemika. Hexamethyleneimine, N,N-dimethylformamide dimethyl acetal, 2-butanone, $p$-toluene sulfonic acid, Sephadex G-10, trifluoroacetic acid, 1,1-carbonyl diimidazole, and potassium phosphate mono- and di-basic were purchased from Sigma-Aldrich Chemical Company. Tertiary amine resin beads were purchased from
Argonaut Technologies, Inc. D-alanyl-D-alanine, L-alanyl-D-alanine and Boc-D-alanine were purchased from Chem-Impex International. D-Thiolactic acid was prepared in this lab by Dr. Ish Kumar.

*Escherichia coli* PBP2 was a generous gift from Dr. H. Adachi of the University of Tokyo. The β-lactamase P99 from *Enterobacter cloacae* was purchased from the Centre for Applied Microbiology and Research (Porton Down, Wiltshire, U.K.) and was used as received. The *Streptomyces* R61 DD-peptidase was generously provided by Dr. J.-M. Frère, of the Université de Liège, Liège, Belgium. Bromopenicillin and mecillinam were generous gifts from Leo Pharmaceutical Company. Enzyme kinetics assays were monitored using a Hewlett Packard 8453 UV Spectrophotometer.
### 2.2 Synthesis and Characterization of 1 – Methods

The synthetic route was adapted from the patent for mecillinam, filed by Leo Pharmaceuticals in Denmark. The synthesis of 1 required only the creation of hexamethyleneiminecarboxaldehyde dimethyl acetal (Scheme 2.1), and its subsequent reaction with D-alanyl-D-alanine (Scheme 2.2).

#### Scheme 2.1 – Synthesis of hexamethyleneiminecarboxaldehyde dimethyl acetal.

**Synthesis of Hexamethyleneiminecarboxaldehyde Dimethyl Acetal**

The synthetic route was adapted from the patent for imidazorifamycins. N,N-dimethylformamide dimethyl acetal (22.3 mL, 157 mmol) and hexamethyleneimine (11.3 mL, 99 mmol) were refluxed for 3 hours. Volatile impurities were removed by a rotary evaporator. The crude product mixture was fractionally distilled at 15 mm Hg, with the desired product distilling between 135 – 150 °C. Product identity and purity was confirmed by $^1$H-NMR (CDCl$_3$): $\delta$ 4.50 (1H, s, CH), 3.48 (1H, s, impurity methanol CH$_3$), 3.33 (6H, s, 2 OCH$_3$s), 2.79 (4H, t, J = 5.7 Hz, 2 NCH$_2$s), 1.59 (8H, br s, 4 ring CH$_2$s) with CDCl$_3$ as the reference at 7.27 ppm (Figure 2.1).
Synthesis of 1: First Attempt

The synthetic scheme was adapted from example 9 of the patent for mecillinam.¹⁶ Hexamethyleneiminecarboxaldehyde dimethyl acetal (590 μL, 3.2 mmol) was added to D-alanyl-D-alanine (400 mg, 2.5 mmol), dissolved in 2.7 mL methanol, and the mixture was stirred for 5 hours at room temperature. The product mixture was filtered through a gravity filter, and dried on a rotary evaporator and further on a vacuum pump. The ¹H-NMR spectrum of the resultant yellow glass showed that all necessary product peaks were present. Crystallization of the compound was attempted as outlined in example 9 of the patent for mecillinam.¹⁶ The crude product was dissolved in minimal 2-butanol, the pH of the solution adjusted to 7.5 by addition of 10% p-toluene sulfonic acid, and the solution was allowed to stand at 5 °C overnight. All crystallization attempts failed.

Synthesis of 1: Second Attempt

In this attempt, triethylamine was added to the reaction mixture as a catalyst as outlined in example 8 of the mecillinam patent.¹⁶ The reaction used the same molar ratios as in the first attempt, but with half the starting materials, and triethylamine (190 μL, 1.4 mmol). The reaction was stirred for 1.5 hours at 5 °C. The product mixture was dried as above, and the ¹H-NMR spectrum showed that all necessary product peaks were again present. Crystallization was attempted as outlined in example 8 of the mecillinam patent.¹⁶ The crude product was dissolved in minimal 2-butanol, the pH of the solution adjusted to 7.5 by addition of triethylamine, and the
solution allowed to stand at 5 °C overnight, but no crystals formed. Further attempts utilized ethylene glycol dimethyl ether, tetrahydrofuran, and diethyl ether as solvents, but again all crystallization attempts failed.

**Synthesis of 1: Third Attempt**

This attempt used the same molar ratios and amounts of materials as the second synthesis, above, but the triethylamine was replaced with resin-bound tertiary amine (400 mg, 1.6 mmol equivalent at 3.9 mmol/g). The reaction mixture was stirred for 2 days at 5 °C. The mixture was then filtered through a sintered glass funnel to remove the tertiary amine resin beads, and the beads were washed with 5 mL more methanol. The combined filtrate was dried on a rotary evaporator and dried further on a vacuum pump overnight. The resultant orange-colored glass weighed 395 mg, and its $^1$H-NMR spectrum showed that all necessary product peaks were again present.

This crude product was dissolved in 1 mL of water and run through a Sephadex G-10 column (radius 0.5 cm, height 20 cm, 100% water eluant). The collected fractions were analyzed by UV-visible spectroscopy. Upon pooling and drying, $^1$H-NMR spectra of all samples were taken in D$_2$O to assess purity. The purest fractions yielded 25.3 mg of 1, with small amounts of impurity still present. This product was dissolved in 200 μL water and run through the Sephadex G-10 column a second time. Fractions were analyzed, pooled, dried and $^1$H-NMR spectra were taken as described above. The purest fractions contained 3.6 mg of 1, and the $^1$H-NMR of this sample showed compound 1, with a small amount of impurity present. This
sample was also analyzed by mass spectrometry, and the sample had the calculated mass of 1: m/z = 270.3.

![Chemical structures](image)

**Scheme 2.2** – Successful synthesis of 1. Note the omission of stereochemistry at the N-terminal alanine α-carbon.

**HPLC Purification of 1**

Attempts to isolate pure 1 required several more syntheses (attempts four through eight), all of which used the same molar ratios as those of attempt 3. The crude product from the eighth synthesis attempt (817.9 mg) was dissolved in water (3 mL) and purified by size exclusion chromatography (Sephadex G-10 column, radius 1 cm, height 25 cm, 100% water eluant). The purest fractions contained 509.4 mg of material, which was > 90% pure 1 as shown by 1H-NMR. This product was dissolved in water to create a 30 mg/mL solution, which was the highest concentration that still
showed good HPLC peak separation. The solution was subsequently filtered by syringe filter, and run through the HPLC.

The elution solvent gradient ran from 98% water (solvent A, containing 0.1% trifluoroacetic acid, TFA) to 88% water over 36 minutes. Solvent B was acetonitrile, containing 0.085% TFA. The flow rate was 3.3 mL/minute, 100 μL of sample solution was injected, and absorbance was monitored at 240 nm. After optimization of the separation (Figure 2.3), the elution profile showed two major peaks, one around 26 minutes and the second around 31 minutes, which were termed 1A and 1B, respectively (Figure 2.2). Each peak was collected separately and freeze-dried, and \(^1\)H-NMR and mass spectra confirmed that both peaks were pure \(\text{I}\).

\(^1\)H-NMR of \(\text{1A}\) (20 mM phosphate D\(_2\)O buffer, pH 7.5): \(\delta\) 7.95 (1H, s, amidine CH), 4.35 (1H, q, \(J = 6.9\) Hz, methine CH), 4.14 (1H, q, \(J = 6.9\) Hz, methine CH), 3.66 (2H, t, \(J = 5.7\) Hz, NCH\(_2\)), 3.50 (2H, t, \(J = 5.7\) Hz, NCH\(_2\)), 1.81 (4H, br s, 2 ring CH\(_2\)s), 1.60 (4H, br s, 2 ring CH\(_2\)s), 1.51 (3H, d, \(J = 6.9\) Hz, ala methyl CH\(_3\)) 1.34 (3H, d, \(J = 6.9\) Hz, ala methyl CH\(_3\)) with the DSS peak as the reference at 0.00 ppm. (Figure 2.4).

\(^\text{1}^\text{H}-\text{NMR of 1B} \) (20 mM phosphate D\(_2\)O buffer, pH 7.5): \(\delta\) 7.94 (1H, s, amidine CH), 4.34 (1H, q, \(J = 6.9\) Hz, methine CH), 4.14 (1H, q, \(J = 7.2\) Hz, methine CH), 3.67 (2H, t, \(J = 5.7\) Hz, NCH\(_2\)), 3.51 (2H, t, \(J = 5.7\) Hz, NCH\(_2\)), 1.81 (4H, br s, 2 ring CH\(_2\)s), 1.60 (4H, br s, 2 ring CH\(_2\)s), 1.54 (3H, d, \(J = 7.5\) Hz, ala methyl CH\(_3\)) 1.35 (3H, d, \(J = 7.2\) Hz, ala methyl CH\(_3\)) with the DSS peak as the reference at 0.00 ppm. (Figure 2.5).
Mass spectra were taken of both 1A and 1B, and both have the calculated mass of 1: m/z = 270.2 (Figures 2.6 and 2.7).

HPLC was used to isolate larger amounts of pure 1A and 1B. In all, approximately 54 mg of crude 1 (> 90% pure) was run through the HPLC. Upon pooling and freeze-drying all the collected fractions, 15.3 mg of pure 1A and 9.7 mg of pure 1B were recovered. This represents a recovery rate of 28% for 1A and 18% for 1B, and indicates that for each 100 μL injection at 30 mg/mL, 0.9 mg of 1A and 0.5 mg of 1B can be isolated. 1H-NMR and mass spec again confirmed the purity of each sample. Both isomers were hard, peach-colored glasses.

**Stereoselective Synthesis of 1A and 1B: First Attempt**

The synthesis used the same molar ratios of all reactants as the third synthesis attempt, as described above, but run on a small scale. L-alanyl-D-alanine (20 mg, 0.13 mmol), tertiary amine resin beads (40 mg, 0.16 mmol equivalent at 3.9 mmol/g) and 0.5 mL methanol were added to a round-bottom flask, which was sealed with a rubber septum and placed in a cold room at 5 °C. Once cold, hexamethyleneimine-carboxaldehyde dimethyl acetal was added to the mixture through the septum via a metal syringe. The reaction was stirred at 5 °C for 6 hours. The mixture was then allowed to come to room temperature and filtered through a sintered glass funnel to remove the tertiary amine resin beads, and the beads were washed with 1 mL more methanol. The combined filtrate was dried on a rotary evaporator and dried further on a vacuum pump overnight. The resultant crude product was used for a 1H-NMR
spectrum, which showed that both isomers were present, as indicated by the presence of two slightly different, overlapping N-terminal methyl doublets. The synthesis was repeated using D-alanyl-D-alanine, and the same result was observed.

**Stereoselective Synthesis of 1A and 1B: Second Attempt**

The synthesis above was repeated, but the reaction vessel was placed in a dry ice/acetone bath at -78 °C. Once cold, hexamethyleneiminecarboxaldehyde dimethyl acetal was added to the mixture through the septum via a metal syringe. The reaction was stirred at -78 °C for 6 hours. The mixture was filtered, and the resin was washed and dried as above. The resultant crude product was used for a $^1$H-NMR spectrum, which showed that only the 1A isomer was present. The synthesis of 1B was the same as that of 1A, but used D-alanyl-D-alanine instead of L-alanyl-D-alanine. The resultant crude product was used for a $^1$H-NMR spectrum, which showed that only the 1B isomer was present, with some degradation products. See Figure 2.9 for a comparison of both spectra.

**Aqueous Degradation of 1A and 1B, Followed by $^1$H-NMR Spectroscopy**

Approximately 2 mg of each isomer (HPLC-purified as described above) was dissolved in a 20 mM phosphate D$_2$O buffer, pH 7.5 containing DSS as the reference at 0 ppm. Degradation was followed by $^1$H-NMR over 8 days (Figures 2.11 – 2.13).
Scheme 2.3 – Stereospecific Synthesis of 1. The 1B isomer is shown.

Synthesis of N-Formyl D-Alanyl-D-Alanine and N-Formyl L-Alanyl-D-Alanine

The procedure was adapted from Sheehan and Yang, 1958. D-Alanyl-D-alanine (or L-alanyl-D-alanine) (100 mg, 0.6 mmol) was dissolved in 96% formic acid (1.31 mL, 35 mmol) and brought to 5 °C, followed by dropwise addition of acetic anhydride (437 μL, 4.7 mmol). The mixture was stirred for 1 hour at 5 °C. Ice water (500 μL) was then added and the mixture was allowed to come to room temperature and dried on a rotary evaporator. The resulting product failed to
recrystallize from water or ethanol as was suggested in the reference, nor would it recrystallize from diisopropyl ether, isopropanol, or any combination of these solvents. Instead, the crude product was dissolved in water to make a 30 mg/mL solution, which was run through the HPLC. The elution solvent gradient was the same as above.

The N-formyl-D-alanyl-D-alanine eluted from the column in 6.4 minutes. Apparently the reaction had not gone to completion, because a significant amount of D-alanyl-D-alanine was observed in the elution profile at 5.2 minutes. $^1$H-NMR of N-formyl-D-alanyl-D-alanine (20 mM phosphate D$_2$O buffer, pH 7.5): $\delta$ 8.07 (1H, s, formyl CH), 4.42 (1H, q, $J = 7.5$ Hz, methine CH), 4.13 (1H, q, $J = 7.5$ Hz, methine CH), 1.40 (3H, d, $J = 6.9$ Hz, ala methyl CH$_3$) 1.33 (3H, d, $J = 7.5$ Hz, ala methyl CH$_3$) with the DSS peak as the reference at 0.00 ppm (Figure 2.14).

The N-formyl-L-alanyl-D-alanine eluted from the column in 7.6 minutes, and a significant amount of L-alanyl-D-alanine was observed in the elution profile at 5.8 minutes. $^1$H-NMR of N-formyl-L-alanyl-D-alanine (20 mM phosphate D$_2$O buffer, pH 7.5): $\delta$ 8.10 (1H, s, formyl CH), 4.43 (1H, q, $J = 7.2$ Hz, methine CH), 4.15 (1H, q, $J = 7.2$ Hz, methine CH), 1.39 (3H, d, $J = 7.2$ Hz, ala methyl CH$_3$) 1.32 (3H, d, $J = 7.5$ Hz, ala methyl CH$_3$) with the DSS peak as the reference at 0.00 ppm (Figure 2.14).
2.3 Synthesis and Characterization of 1 – Results

The synthetic route was adapted from the patent for mecillinam, filed by Leo Pharmaceuticals in Denmark. After several trial attempts, described above in Section 2.2 and discussed further below, a successful, stereospecific total synthesis of 1 was achieved and is shown here as Scheme 2.4.

![Scheme 2.4 – Successful, stereospecific synthesis of 1. The 1B isomer is shown.](image)

**Synthesis of Hexamethyleneiminecarboxaldehyde Dimethyl Acetal**

The synthesis and distillation of hexamethyleneiminecarboxaldehyde dimethyl acetal was straightforward, but it is important to note that the compound is very sensitive to water, and must be stored under dry nitrogen at -70 °C. The yield is not quantitative because all heteroatom substituents on the acetal carbon are labile and
one might expect that, with two different secondary amines present in the reaction mixture, nine different compounds of three types might be products of the reaction (two amide acetals, three ester aminals and four orthoamides). The existence of these side reactions has been demonstrated\textsuperscript{19} and explains why the yield of this transamination reaction is often lower than 70%.

\textbf{Figure 2.1} \textsuperscript{1}H-NMR of hexamethyleneiminecarboxaldehyde dimethyl acetal in CDCl\textsubscript{3}
Synthesis of Compound 1

The very first attempted synthesis of 1 appeared to be successful, however, its purification proved to be very challenging. At first, the existence of impurities seemed to be the only problem, and the attempted remedies included switching to resin-bound tertiary amine catalyst, which is easily removable by filtration, and purification of the crude product by size-exclusion chromatography. However, even after these steps were taken, the product remained impure, and purification by HPLC was turned to as a last resort. After extensive experimentation with different solvents and gradients, the HPLC elution profile clearly showed that two major product peaks were present, which were termed 1A and 1B (Figure 2.2). Optimization of peak separation was achieved by altering the solvent gradient, which allowed the two peaks to be collected individually (Figure 2.3).

![Figure 2.2](image)

**Figure 2.2** – Final HPLC elution profile showing peaks A and B.  
1A eluted around 26 minutes and 1B eluted around 31 minutes.
Figure 2.3 – Optimization of HPLC peak separation for 1. All gradients started with 98% water, 2% acetonitrile and gradually decreased in percentage of water over 36 minutes to: a) 80%; b) 88%; c) 92%; d) 94%; e) 96%. Gradient (d) was chosen to isolate samples of 1A and 1B for subsequent experiments.
The 509 mg of product obtained from the size-exclusion column was > 90% pure 1. This suggests that the yield of the compound 1 synthesis at 5 °C was around 70%. This compares very well with the 70 – 90% yields reported in the synthesis of mecinllinam. However, the final isolated yields of pure 1A and 1B were only 28% and 18%, respectively. The HPLC purification process was responsible for this decrease in the apparent yield.

The material injected into the HPLC was > 90% pure 1, and the elution profile clearly shows that the 1A and 1B peaks are indeed the dominant components of the mixture, even when including the materials released from the column in the acetonitrile flush (36 minutes to 50 minutes in Figure 2.2). However, only around 50% of the injected material was recovered from the HPLC as pure product. This indicates that a significant amount of product is lost somewhere in the HPLC purification process, but efforts to determine the nature of this substantial column tax were not successful.

Each product was independently shown to be 1 by 1H-NMR and mass spectrometry (Figures 2.4 – 2.7), which suggests that the reaction was successful, but produced two isomers. The NMR spectra of the two isomers were nearly identical, but when taken in a 20 mM phosphate, pH 7.5 D2O buffer, with a DSS reference peak at 0 ppm, one small difference became clear between the two isomers. The shift of the N-terminal methyl doublet in the 1A isomer is 0.03 ppm further upfield than the corresponding peak in the 1B isomer (Figure 2.8).
Figure 2.4 – $^1$H-NMR spectrum of 1A in 20 mM phosphate D$_2$O buffer, pH 7.5.

The resolution of the spectrum was poor due to instrument difficulties.
Figure 2.5 – $^1$H-NMR spectrum of 1B in 20 mM phosphate D$_2$O buffer, pH 7.5
Figure 2.6 – Mass spectrum of 1A

Figure 2.7 – Mass spectrum of 1B
Figure 2.8 – Detail of the difference in N-Terminal methyl shifts between 1A and 1B.
Top – A isomer; Middle – B isomer; Bottom – crude, unpurified product mixture.
With two clear differences between the isomers established (HPLC retention time and the N-terminal methyl doublet NMR peak), investigation of the nature of the difference between them began. The first possibility explored was that they were diastereomers, and to investigate this option the synthesis was repeated using L-alanyl-D-alanine. When the synthesis was run at °5 C, the product mixture contained the same two isomers, as evidenced by the HPLC elution profile. This result strongly suggests that the two isomers are indeed diastereomers.

Assignment of Absolute Stereochemistry to 1A and 1B

In order to assign absolute stereochemistry to both isomers, several experiments were performed. First, an R61 DD-peptidase ¹H-NMR experiment was performed, because presumably only the D-alanyl-D-alanine isomer would be a substrate for the enzyme. Neither isomer, however, seemed to be a substrate of the R61 DD-peptidase. Next, samples of both isomers were subjected to acid hydrolysis in order to free the two alanines, and then a quantitative L-alanine assay was performed, but neither isomer seemed to contain any L-alanine. Along a similar vein, the acid-hydrolyzed samples were used in a D-amino acid oxidase assay, but neither isomer seemed to contain even one full equivalent of D-alanine. It is now understood that acid hydrolysis was not effective at cleaving the amidine bond, and so the N-terminal alanine was not available to the enzyme used in the assay. The experiments were repeated using samples of both isomers which were base-hydrolyzed, but with the same result. It is now understood that base hydrolysis of the amidine bond yields
the N-formyl alanine, which presumably was not a good substrate for either enzyme assay. Finally, it was discovered that hydrazine cleaves amidine bonds to yield a free N-terminus\textsuperscript{20} (rather than N-formylated) and this reaction was studied by $^1$H-NMR in D$_2$O. Hydrazinolysis was indeed observed to cleave the amidine bond in both isomers, however, the N-terminal methine exchanged quantitatively with deuterium in the process, as evidenced by the loss of the methine quartet and the collapse of the N-terminal methyl doublet to a singlet in the $^1$H-NMR spectrum. Presumably, stereochemistry at the N-terminal alanine $\alpha$-carbon was lost in the hydrazinolysis process, as exchange with deuterium requires epimerization. All the above attempts to identify the absolute stereochemistry of the two isomers of compound 1 were not successful.

At this point, the only clue as to which diastereomer was which was the relative difference in their HPLC retention times. Separation of diastereomeric peptides and their derivatives is known to be achievable by HPLC.\textsuperscript{21-23} The literature seems to be in unanimous agreement that homochiral peptides are more hydrophilic than heterochiral peptides, and always elute from HPLC columns faster than heterochiral peptides. This was confirmed in this work with L-alanyl-D-alanine and D-alanyl-D-alanine as well as their N-formyl derivatives. In each case, the homochiral peptide molecule eluted faster than the heterochiral one. This trend would assign the A isomer as the DD diastereomer and the B isomer as the LD diastereomer. In the present case, however, this assignment was eventually proven to be incorrect. This work may be the first case of a homochiral peptide derivative that exhibits a
longer HPLC retention time than its heterochiral diastereomer. This aberrant behavior may be due to the unusual nature of the amidine side chain.

Finally, a stereospecific synthesis was achieved. When the synthesis was run at -78 °C, only one set of N-terminal methyl doublets appeared in the crude product, and only one isomer peak was dominant in the HPLC elution profile. When L-alanyl-D-alanine was used as the starting material, the crude product $^1$H-NMR showed only peaks that correspond to the A isomer, and the HPLC elution profile of the crude product showed that mostly the A peak is present. When D-alanyl-D-alanine was used, the crude product $^1$H-NMR showed only the B isomer peaks, and the HPLC elution profile showed that mostly the B peak was present. See Figure 2.9 for the NMR comparisons, and Figure 2.10 for the HPLC elution profile comparison.

Assignment of absolute stereochemistry to 1A and 1B
Figure 2.9 – Details of NMR spectra of HPLC-purified 1A and 1B compared to the NMR spectra of crude product from stereospecific syntheses. The stereospecific synthesis using L-alanyl-D-alanine yielded remarkably pure product, while some degradation products were visible in the D-alanyl-D-alanine product.
Figure 2.10 – Comparison of HPLC elution profiles. Stereospecific synthesis using L-alanyl-D-alanine yielded mostly the A peak, while the synthesis using D-alanyl-D-alanine yielded mostly the B peak. When the reaction was run at 5 °C, both isomers are seen regardless of the stereochemistry of the starting material.
Aqueous Degradation of 1A and 1B, Followed by $^1$H-NMR Spectroscopy

Mecillinam has been shown to yield many different degradation products on incubation in aqueous solution, and different pathways prevail under acidic and basic conditions. Therefore it was important to study the degradation of 1 under carefully controlled, well-defined conditions. $^1$H-NMR was chosen to follow degradation over a period of weeks to months, and the solvent selected was a 20 mM phosphate D$_2$O buffer, pH 7.5, similar to that used for the enzyme kinetics assays. Figure 2.11 displays the changes in the $^1$H-NMR spectrum of 1B over time in this buffer.

The degradation of mecillinam exhibits both epimerization at the carbon adjacent to the amidine, and amidine hydrolysis, yielding hexamethyleneimine and N-formyl-penicillanic acid. Degradation of compound 1, therefore, might be expected to yield hexamethyleneimine and N-formyl-alanyl-alanine. To confirm the identity of the degradation products, N-formyl-D-alanyl-D-alanine and N-formyl-L-alanyl-D-alanine were synthesized and purified, and their NMR spectra were taken in the D$_2$O buffer. See Figure 2.12 for a comparison of their spectra with that of degraded 1.

The complete degradation of 1 at pH 7.5 did indeed yield hexamethyleneimine and N-formyl-dipeptide, but each diastereomer degraded to a 50/50 mixture of N-formyl-D-alanyl-D-alanine and N-formyl-L-alanyl-D-alanine, indicating that quantitative epimerization occurred during the degradation of 1. In the N-formyl dipeptides the N-terminal methine had quantitatively exchanged with deuterium, and the N-terminal alanine methyl doublet had collapsed to a singlet, accordingly.
Figure 2.11 – Degradation of 1B in 20 mM phosphate D$_2$O buffer, pH 7.5. \(^{1}\)H-NMR of 1B at 0 (top), 96 (middle) and 192 hours (bottom). The spectrum of fully degraded 1A is identical to that of 1B at 192 hours.
Figure 2.12 – Comparison of $^1$H-NMR spectra of degraded 1 (either isomer) with N-formyl-L-alanyl-D-alanine and N-formyl-D-alanyl-D-alanine. Note the missing methine quartet (arrow) and the methyl signal at 1.4 ppm collapsed to a singlet.
In 20 mM phosphate buffer, pH 7.5, the half life of 1A was determined to be 126 hours, and the half life of 1B was determined to be 65 hours (Figures 2.13 and 2.14). The degradation was followed using the disappearance of the amidine peak at 7.95 ppm. These values compare well with the half life of mecillinam, which is approximately 100 hours at pH 6.\textsuperscript{24}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure2.13.png}
\caption{Disappearance of 1A, as followed by the amidine peak at 7.95 ppm.}
\end{figure}
Figure 2.14 – Disappearance of $1B$, as followed by the amidine peak at 7.95 ppm.

In aqueous solution at pH 6 and at pH 10, mecillinam has been reported to epimerize at the 6-position before both lactam ring-opening and hydrolysis of the amidine bond.\textsuperscript{24} Degradation of compound 1 was expected to occur in a similar fashion to that of mecillinam, with separate, different rates for epimerization and amidine hydrolysis. However, no epimerization was observed in the starting compound, and the N-formyl dipeptide product appears in a 50/50 mixture of diastereomers even at their first appearance. Thus, the NMR spectra seemed to show
that amidine hydrolysis (and accompanying release of hexamethylenimine) somehow occurred in a concerted fashion with the epimerization at the N-terminal alanine α-carbon. Therefore, at pH 7.5 at least, compound 1 degrades to similar products as does mecillinam, but via a different mechanism.

Proposed Mechanism of Degradation of 1

Simple hydrolysis of the amidine bond was expected in the degradation of 1, and would explain the release of hexamethylenimine, but attack of water directly at the amidine carbon cannot account for the observation that amidine hydrolysis occurred in a concerted fashion with epimerization. An intramolecular cyclization, as shown below in Scheme 2.5, would, however, account for all the observed products. Intramolecular cyclization to form 4 would make the methine acidic, and cause it to exchange with deuterium in a fast manner via 5. This mechanism could explain the epimerization and the disappearance of the methine proton signal. Collapse of 4 or 5 back to the starting material 1 must be slow relative to hydrolysis of the cyclized intermediate, at least in the particular buffer used, because no epimerization of the starting products is observed. This intramolecular cyclization mechanism is similar to the mechanism of O → N acyl migrations.²⁵ It is interesting to note that no oxazole formation was observed. Attack of the water molecule must therefore rapidly occur at the imine carbon of 4 (Scheme 2.6).
Scheme 2.5 – Possible Mechanism of Intramolecular Cyclization of 1. Epimerization may occur because the methine proton in 4 would exchange with deuterium via 5.
Scheme 2.6 – Hydrolysis of the Cyclized Intermediate 4 Would Yield Hexamethyleneimine and a Diastereomeric Mixture of N-Formyl Dipeptide.

This mechanism may also account for the epimerization that occurred in the synthesis of 1. If the attack of methanol on the cyclized intermediate 4 were slow, then the intermediate would have time to convert back to the starting material 1, yielding fully epimerized compound 1, which was the product observed when the reaction was run at room temperature and at 5 °C. Formation of the cyclized intermediate 4 may be unfavorable at -78 °C, which would explain why stereochemistry was largely retained when the reaction was run at this low temperature. This mechanism will be discussed further in the Discussion (Section 7).
III. SYNTHESIS AND CHARACTERIZATION OF 2
3.1 Synthesis and Characterization of 2 – Methods

The synthesis of 2 required creation of the Boc-protected D-alanyl-thioglycolic acid (Scheme 3.1), followed by cleavage of the Boc protecting group (Scheme 3.2), and then formation of the amidine bond (Scheme 3.4).

Scheme 3.1 – Formation of Boc-D-alanyl-thioglycolic acid.

Synthesis of Boc-D-Alanyl-Thioglycolic Acid

The procedure was adapted from the undergraduate thesis of Gabriel Soto, 1992.26 Boc-D-alanine (3.73 g, 20 mmol) and 1,1'-carbonyl diimidazole (CDI) (3.20 g, 20 mmol) were dissolved in 120 mL dry THF and allowed to stir at 5 °C for one hour. The solution was clear and colorless at this point. Then a solution of mercaptoacetic acid (thioglycolic acid) (1.51 mL, 22 mmol) in 10 mL dry THF, was added to the Boc-D-ala/CDI mixture. The reaction mixture turned from a bright neon green color to clear and colorless after about six hours. The mixture was dried on a rotary evaporator, taken up in 100 mL ethyl acetate, and washed twice with 100 mL 10% citric acid solution and twice with 100 mL water. The product remained in the ethyl acetate layer, which was collected, dried over MgSO₄, filtered through a gravity filter, and dried on a rotary evaporator, and further on a vacuum pump overnight. The product was a white solid at this point, and its weight was 3.26 g (67 % yield).
Recrystallization from benzene occurred when a solution was left at 5 °C overnight. The crystals were white, and mostly adhered to the bottom of the flask in a large sheet. Their mass was 2.13 g (44 % final overall yield). The purity and identity of the crystals was confirmed by $^1$H-NMR (CDCl$_3$): $\delta$ 4.95 (1H, br s, NH), 4.45 (1H, m, CH), 3.73 (2H, q, J = 15 Hz, CH$_2$), 1.47 (9H, s, Boc), 1.42 (3H, d, J = 6.9 Hz, CH$_3$), with the chloroform peak as the reference at 7.27 ppm (Figure 3.1). The melting point of the Boc-D-alanyl-thioglycolic acid crystals was between 108 – 110 °C.

**Synthesis of 2: First Attempt**

Boc-group deprotection proceeds readily in the presence of acid. Boc-D-alanyl-thioglycolic acid (100 mg, 0.4 mmol), was suspended in 1 mL methylene chloride in a 15 mL round bottom flask and stirred on ice for 10 minutes. TFA, 10 mL, was then added and the mixture was allowed to come to room temperature, stirring for two hours. The solution was dried on a rotary evaporator, 1 mL of benzene was added, and the solution was dried again, and dried further on a vacuum pump overnight, yielding the product as a colorless glass.

![Scheme 3.2 – Boc cleavage of Boc-D-alanyl-thioglycolic acid.](image)
The amidine formation was attempted as outlined in the successful synthesis of 1. The colorless glass from above was taken up in 1.2 mL of methanol, and tertiary amine resin beads (250 mg, 1.0 mmol equivalent at 3.9 mmol/g) were added to the mixture at room temperature. Finally hexamethyleneiminecarboxaldehyde dimethyl acetal (91 μL, 0.5 mmol) was added, and the mixture was sealed with a rubber septum and moved to the cold room and stirred for 48 hours. The mixture was then filtered through a sintered glass funnel to remove the tertiary amine resin beads, and the beads were washed with 5 mL more methanol. The combined filtrate was dried on a rotary evaporator and dried further on a vacuum pump overnight. The resultant beige-colored glass weighed 137 mg.

The crude reaction mixture was dissolved in 4.5 mL water to make a 30 mg/mL solution, which was subsequently filtered by syringe filter, and run through the HPLC using the same gradient as was used above for compound 1 (Section 2.2). The flow rate was 3.3 mL/minute, 150 μL of sample solution was injected, and absorbance was monitored at 240 nm. The elution profile showed one major peak which eluted around 20.5 minutes (Figure 3.2). The peak was collected and freeze-dried, but the $^1$H-NMR spectrum of the residual solid showed that the product had methanolyzed, yielding the methanolysis product, 6. $^1$H-NMR (D$_2$O): δ 7.92 (1H, s, amidine CH), 4.44 (1H, q, J = 6.9 Hz, methine CH), 3.77 (3H, s, OCH$_3$), 3.64 (2H, t, J = 5.7 Hz, NCH$_2$), 3.49 (2H, t, J = 5.7 Hz, NCH$_2$), 1.78 (4H, br s, 2 ring CH$_2$s), 1.59 (4H, br s, 2 ring CH$_2$s), 1.53 (3H, d, J = 6.9 Hz, ala methyl CH$_3$) with the D$_2$O peak as the reference at 4.80 ppm (Figure 3.3).
Scheme 3.3 – Unsuccessful first synthesis of 2 – product methanolyzed.

Synthesis of 2: Second Attempt

The deprotection of Boc-D-alanyl-thioglycolic acid (100 mg, 0.4 mmol), was achieved as described above. In an effort to prevent or reduce methanolsysis during the amidine formation, the amount of tertiary amine resin beads was reduced to one molar equivalent for this synthesis attempt. D-Alanyl-thioglycolic acid, obtained as described above as a colorless glass, was taken up in 413 μL of methanol, and tertiary amine resin beads (97 mg, 0.4 mmol equivalent at 3.9 mmol/g) were added to the mixture at room temperature. Finally, hexamethyleneiminecarbox-aldehyde dimethyl acetal (66 μL, 0.4 mmol) was added, the mixture was sealed with a rubber septum, moved to the cold room and stirred for 48 hours. The mixture was then filtered.
through a sintered glass funnel to remove the tertiary amine resin beads, and the beads were washed with 5 mL more methanol. The combined filtrate was dried on a rotary evaporator and further on a vacuum pump overnight. The resultant beige-colored glass weighed 84 mg.

The crude product was dissolved in 2.78 mL water to make a 30 mg/ml solution, which was subsequently filtered by syringe filter, and run through the HPLC as described above. The elution profile showed one large peak at 22.8 minutes, but the peak was irregular in shape, with smaller peaks contained in it. The peak was collected in two halves and freeze-dried, and the $^1$H-NMR spectrum showed that the product had again methanolyzed. The identity of the methanolysis product 6 was confirmed by mass spectrometry: m/z = 213.2 (Figure 3.4).

**Synthesis of 2: Third Attempt**

The deprotection of Boc-D-alanyl-thioglycolic acid (100 mg, 0.4 mmol), was achieved as described above. In order to prevent methanolysis during amidine formation, a more bulky solvent was used. Boc-D-alanyl-thioglycolic acid was insoluble in chloroform, and the freezing point of tert-butyl alcohol was far too high to be used at -78 °C. Eventually, isopropanol emerged as the solvent of choice, as its hydroxyl is much more sterically hindered than that of methanol. D-alanyl-thioglycolic acid, as a colorless glass, was taken up in 1 mL of isopropanol, and tertiary amine resin beads (97.4 mg, 0.4 mmol equivalent at 3.9 mmol/g) were added to the mixture. This mixture was capped with a rubber septum and placed in a dry
ice/acetone bath at -78 °C. Finally, hexamethyleneiminecarboxaldehyde dimethyl acetal (66 μL, 0.4 mmol) was added through the septum via a Hamilton syringe, and the mixture was allowed to stir at -78 °C for 6 hours. The mixture was then allowed to come to room temperature, quickly filtered through a sintered glass funnel to remove the tertiary amine resin beads, and the beads were washed with 5 mL isopropanol. The combined filtrate was dried on a rotary evaporator and dried further on a vacuum pump overnight. The resultant glass weighed 148 mg.

Scheme 3.4 – Successful synthesis of 2.
The crude product was dissolved in 4.92 mL water to make a 30 mg/ml solution, which was subsequently filtered by syringe filter, and run through the HPLC as described above. The elution profile showed two major peaks, the smaller one at 22.4 minutes and the larger one at 26.7 minutes. Both peaks were collected and freeze-dried, and their $^1$H-NMR spectra were taken. The compound isolated from the first peak could not be identified, but the compound from the second peak (26.7 minutes) had all the necessary signals to be $\mathbf{2}$. $^1$H-NMR in 20 mM phosphate D$_2$O buffer, pH 7.5: $\delta$ 8.03 (1H, s, amidine CH), 4.52 (1H, q, $J = 7.5$ Hz, methine CH), 3.70 (2H, t, $J = 6.0$ Hz, NCH$_2$), 3.62 (2H, s, SCH$_2$), $\delta$3.57 (2H, m, $J = 6.3$ Hz, NCH$_2$), 1.85 (4H, br m, 2 ring CH$_2$s), 1.63 (4H, br m, 2 ring CH$_2$s), 1.56 (3H, d, $J = 6.9$ Hz, ala methyl CH$_3$) with the DSS as the reference at 0.00 ppm (Figure 3.6). Product identity was confirmed by mass spectrometry: m/z = 273.1 (Figure 3.7).

To prepare larger amounts of pure $\mathbf{2}$, the remaining crude 30 mg/mL solution was freeze-dried and made into a 60 mg/mL solution. Over the course of ten runs, approximately 90 mg of crude $\mathbf{2}$ was run through the HPLC. Upon pooling and freeze-drying all the collected product fractions, however, only 4.2 mg of pure $\mathbf{2}$ was recovered.

**Synthesis of $\mathbf{2}$: Fourth Attempt**

The successful synthesis above was repeated using double the amount of starting material, Boc-D-alanyl-thioglycolic acid (200 mg, 0.8 mmol), and the resultant crude product amounted to 339 mg. The crude product was dissolved in 5.66
mL water to make a 60 mg/mL solution, which was subsequently filtered by syringe filter, and run through the HPLC as described above. The elution profile was slightly different from that of the third synthesis. A small impurity peak appeared just before the large putative product peak, and the resolution of these peaks was poor, prompting a re-optimization of the separation. After several trial runs, a new gradient of 98\% H₂O to 92\% H₂O over 36 minutes was chosen. In this new gradient, the large product peak eluted around 27.5 minutes. The elution profile is shown as Figure 3.5.

In all, 15 more HPLC runs were performed and all product peaks were collected, pooled and freeze-dried. The dissolved product was syringe-filtered before final lyophilization in a pre-weighed vial. Of the approximately 153 mg of total crude product injected into the HPLC, 28.7 mg of pure 2 were recovered. This represents a recovery rate of 19\%, and indicates that for each 150 μL injection of crude product at 60 mg/mL, approximately 1.7 mg pure 2 can be recovered. This large amount of product was confirmed to be 2, intact and pure by both ¹H-NMR and mass spectrometry: m/z = 273.2. The product was a hard, sticky, peach-colored glass.

Identity of 2 Further Confirmed by Base Hydrolysis: An NMR Experiment

Four NMR samples were prepared, each with 690 μL D₂O, 10 μL NaOD (10 M). Of these four tubes, the first two contained 10 μL of reference compound, methanol and thioglycolic acid, respectively. The second two tubes each contained approximately one milligram of test compound, one with the methanolysis product 6, and the final tube with pure compound 2. The samples of 2 and 6 were obtained from
the preparative HPLC runs. The solution in each tube was confirmed to be basic (pH > 10), as indicated by pH paper, except for the thioglycolic acid control tube, which became basic after an additional 10 μL of NaOD was added. ¹H-NMR spectra were obtained for each sample. The resulting spectra are shown as Figures 3.8 and 3.9.

**Aqueous Degradation of 2, Followed by ¹H-NMR Spectroscopy**

Approximately 2 mg of 2 was dissolved in a 20 mM phosphate D₂O buffer, pH 7.5 containing DSS as the reference at 0 ppm. Degradation was followed by ¹H-NMR over 6 days (Figures 3.12 – 3.15). The fully degraded compound 2 ¹H-NMR spectrum in 20 mM phosphate D₂O buffer, pH 7.5: δ 7.86 (1H, s, amidine CH), 4.16 (1H, q, J = 7.8 Hz, methine CH), 3.65 (2H, t, J = 6 Hz, NCH₂), 3.58 (<1H, s, unknown, could be dithio-diglycolic acid), 3.50 (2H, t, J = 5.6 Hz, NCH₂), 3.29 (2H, s, free thioglycolic CH₂), 1.81 (4H, br s, 2 ring CH₂s), 1.60 (4H, br s, 2 ring CH₂s), 1.49 (3H, d, J = 7.5 Hz, ala methyl CH₃) with the DSS as the reference at 0.00 ppm (Figure 3.10).

**Isolation and Characterization of Amidino-Alanine 7**

In order to obtain a pure sample of the amidino-alanine 7, a 200 μL aliquot of crude 2 (30 mg/mL) was allowed to stand at room temperature in an eppendorf tube for five days. This sample was then run through HPLC, using the same gradient as was used above in the first three synthesis attempts. The elution profile had changed such that the compound 2 peak had nearly disappeared, while one impurity peak,
which eluted around 17 minutes, grew larger. This peak was collected and freeze-dried, and characterized by $^1$H-NMR and mass spectrometry. $^1$H-NMR spectrum in 20 mM phosphate D$_2$O buffer, pH 7.5: δ 7.85 (1H, s, amidine CH), 4.10 (1H, q, J = 7.2 Hz, methine CH), 3.64 (2H, t, J = 6 Hz, NCH$_2$), 3.50 (2H, t, J = 6 Hz, NCH$_2$), 1.81 (4H, br s, 2 ring CH$_2$s), 1.60 (4H, br s, 2 ring CH$_2$s), 1.48 (3H, d, J = 7.2 Hz, alan methyl CH$_3$) with the DSS as the reference at 0.00 ppm (Figure 3.12). Product identity was confirmed by mass spectrometry: m/z = 199.2. (Figure 3.11).

![Amidino-alanine 7](image)
3.2 Synthesis and Characterization of 2 – Results

Compound 2 was prepared as shown in Scheme 3.5. The synthesis first required creation of the Boc-protected D-alanyl-thioglycolic acid from Boc-D-alanine and thioglycolic acid. After deprotection of the Boc group, the reactive amine was condensed with hexamethyleneiminecarboxaldehyde dimethyl acetal to afford 2.

Scheme 3.5 – Successful synthesis of 2.

Synthesis of Boc-D-Alanyl-Thioglycolic Acid

The synthesis of Boc-D-alanyl-thioglycolic acid was successful. Its recrystallization, however, was incomplete in the first attempted synthesis. Apparently, in order for effective recrystallization to occur, pure, freshly-purchased thioglycolic acid is required, as was used in the second synthesis. The yield of recrystallized material from that second synthesis was 44%. The crystals were colorless but opaque and formed large sheets. $^1$H-NMR confirmed the purity of the crystals (Figure 3.1).
Figure 3.1 – $^1$H-NMR spectrum of Boc-D-alanyl-thioglycolic acid in CDCl$_3$
**Compound 2 is Susceptible to Methanolysis**

The first two syntheses of 2 were run with methanol as the solvent. It was soon discovered that the thiolester bond was readily methanolyzed, yielding the methanolysis product 6, which was characterized by $^1$H-NMR and mass spectrometry. Most notably the $^1$H-NMR shows a methoxy singlet at 3.57 ppm and the thioglycolic methylene peak is missing (Figure 3.3). Mass spectrometry confirmed that the product had methanolyzed: m/z = 213.2 (Figure 3.4).

![Methanolysis Product 6](image)

Further proof was found in that methanolysis also occurred in the synthesis of compound 3 (Section 4.2), yielding exactly the same methanolysis product 6. The methanolysis products from 2 and 3 had identical HPLC retention times, $^1$H-NMR and mass spectra.

Interestingly, methanolysis seemed to occur preferentially over hydrolysis. This became evident when the mass spectrometry results for 2 were returned from the Mass Spectrometry Resource at UIUC. Despite the fact that the synthesis had been run in isopropanol, the mass spectrum showed it to be the methanolysis product. Upon inquiring, it was discovered that the mass spectrometry technician had
dissolved the sample in methanol. The same sample was sent again and run in acetonitrile instead, and it was indeed intact 2. The amount of time the samples spent in the methanol before mass spectral analysis surely must have been less than the time they spent in aqueous solution in the HPLC and during the freeze-drying process. Therefore, the thiolester bond seems to be more susceptible to methanolysis than hydrolysis.

Figure 3.2 – HPLC elution profile of the crude product of the unsuccessful first synthesis of 2. The peak around 20.5 minutes is the methanolysis product 6.
Figure 3.3 – $^1$H-NMR spectrum of methanolysis product 6
Successful Synthesis of 2

With methanol readily attacking the thiolester, subsequent reactions employed a solvent with a more sterically hindered hydroxyl, in the hope that this would prevent attack at the thiolester bond. Also, the third and fourth synthesis attempts were run at -78 °C in an effort to prevent any epimerization that may occur at the alanine α-carbon. Eventually, isopropanol emerged as the best solvent choice as its hydroxyl group is far more sterically hindered than that of methanol and it is still liquid at -78 °C. Under these new conditions, the reaction was successful. The crude
product was purified by HPLC without the prior purification by size-exclusion column chromatography. The apparent yield of pure 2 from the HPLC was 19%, which is lower than the total recovery rate of compound 1. Assuming a column tax similar to that observed in the HPLC purification of 1, this result would imply the synthesis of 2 proceeded with a yield in the range of 30 – 50%. A lower yield is not surprising, given that the thiolester bond is susceptible to hydrolysis and alcoholysis, particularly in the presence of the tertiary amine base. The elution time of 2 from the HPLC, using the gradient described above for the fourth synthesis (Section 3.1), was around 27.5 minutes (Figure 3.5). The identity of 2 was confirmed by $^1$H-NMR (Figure 3.6), the NMR Base Hydrolysis Experiment (discussed below), and by mass spectrometry: m/z = 273.1 (Figure 3.7).

![Figure 3.5 – HPLC elution profile of crude 2 from the fourth synthesis attempt. Compound 2 eluted around 27.5 minutes.](image-url)
Figure 3.6 – $^1$H-NMR of 2 in 20 mM phosphate D$_2$O buffer, pH 7.5. The spectrum shows a small amount of unknown impurity (marked ?) and a small amount of the degradation product (marked *).
Identity of 2 Further Confirmed by Base Hydrolysis: An NMR Experiment

This experiment was performed to prove that the compounds that had been isolated from the HPLC were indeed 2, and its methanolysis product 6. As discussed earlier, amidine hydrolysis in basic conditions has been studied in mecillinam, and the products of hydrolysis were hexamethyleneimine and 6-formamidopenicillanic acid. Therefore, if 6 were in the NMR tube, the addition of NaOD would be expected to cause both the amidine and the methyl ester to hydrolyze, yielding hexamethyleneimine, N-formyl-alanine and methanol (Scheme 3.6). In the control tube containing
only pure methanol, the CH$_3$ singlet appeared at 3.34 ppm. The sample isolated from the second attempted synthesis of 2 yielded a sharp singlet at 3.34 ppm upon hydrolysis (Figure 3.8), indicating that the second attempted synthesis had indeed created the methanolysis product 6, rather than the desired product.

Similarly, if 2 were in the NMR tube, the addition of NaOD would be expected cause the hydrolysis of the amidine and the thiolester, yielding hexamethylenimine, N-formyl-alanine and thioglycolic acid (Scheme 3.6). In the given basic D$_2$O conditions, the pure thioglycolic acid CH$_2$ control peak appeared at 3.00 ppm. The sample isolated from the third attempted synthesis of 2 yielded a sharp singlet at 2.96 ppm upon hydrolysis, indicating it indeed had started as 2. The chemical shift was not exactly at 3.00 ppm, probably because of a slight difference in pH between the tubes. This result strongly suggests that the third attempted synthesis of 2 was successful.

As expected, the N-formyl-alanine was produced from the hydrolysis of both 2 and 6. The alanine methyl appears as the doublet at 1.35 ppm and the N-formyl proton appears at the singlet at 8.02 ppm. Interestingly, the methine quartet was not visible in either spectrum. The disappearance of the methine could indicate that epimerization and quantitative exchange with deuterium occurred during the degradation or it could simply be hidden under the water peak or unresolved from the noisy baseline. Also, in both sample tubes, the hexamethyleneimine ring was released as expected, appearing as the triplet at 2.74 ppm and the broad multiplet at 1.58 ppm.
Scheme 3.6 – Expected hydrolysis products of 2 and 6 in basic aqueous solution
Figure 3.8 – Comparison of the NMR spectrum of methanol (top) with that of hydrolysis products from compound 6 (bottom). The hydrolysis of 6 clearly releases methanol, hexamethylenimine, and N-formyl alanine.
Figure 3.9 – Comparison of the NMR spectrum of thioglycolic acid (top) with that of hydrolysis products from compound 2 (bottom). The hydrolysis of 2 releases thioglycolic acid, hexamethyleneimine, and N-formyl alanine.
Aqueous Degradation of 2, Followed by $^1$H-NMR Spectroscopy

The NMR spectrum of fully degraded 2 is shown below as Figure 3.10. The aqueous degradation of 2 appeared to give rise to free thioglycolic acid, implying hydrolysis of the thiolester bond. However, unlike the degradation of compound 1, no hexamethyleneimine was released, and the amidine bond remained intact throughout the degradation process and after degradation had gone to completion. This result would suggest that the degradation of compound 2 consisted only of hydrolysis of the thiolester bond, yielding thioglycolic acid and the amidino-alanine compound 7 as degradation products (Scheme 3.7).

**Figure 3.10** – $^1$H-NMR of 2 completely hydrolyzed after 144 hours in 20 mM phosphate D$_2$O buffer, pH 7.5
Scheme 3.7 – Hydrolysis of 2 in 20 mM phosphate buffer, pH 7.5 yields thioglycolic acid and compound 7, amidino-alanine.

To confirm the identity of the degradation products, NMR spectra were taken of thioglycolic acid and the amidino-alanine 7. Compound 7 was HPLC-purified from a sample of crude, degraded 2, and its identity was confirmed by NMR and mass spectrometry (Figure 3.11). NMR spectra of thioglycolic acid, 7, and degraded 2 were all taken in the 20 mM phosphate D₂O buffer, pH 7.5. The comparison of their spectra is shown as Figure 3.12.
Figure 3.11 – Mass spectrum of amidino-alanine 7
Peaks in the NMR spectrum of the fully degraded compound were indeed consistent with those of the amidino-alanine 7 and free thioglycolic acid (Figure 3.12). Of note, the chemical shift of the free thioglycolic acid methylene moved increasingly downfield as the amount of free thioglycolic acid increased over time, which is most likely an effect of the decreasing pH as more thioglycolic acid was released during hydrolysis. The chemical shift of the thioglycolic acid control is also further downfield (by 0.05 ppm) because the sample concentration was greater than that in the tube containing compound 2, and thus the pH was lower.
The remarkable stability of the amidine bond of compound 2 and its degradation product 7 stands in stark contrast to the hydrolytic susceptibility of the amidine bond of compound 1. Indeed, compound 7 seems to be infinitely stable in aqueous solution at pH 7.5, showing no change in its NMR spectrum even after 3 months in the tube. It is likely that the amidine bonds of these compounds remains intact because they are incapable of forming the cyclized intermediate described in the Discussion (Section 7).

**Figure 3.13** – $^{1}$H-NMR of 2 at 0 (top), 24 (middle) and 96 hours (bottom) in 20 mM phosphate D$_2$O buffer, pH 7.5
The changes in the NMR spectrum of 2 over time are shown in Figure 3.13. The half life of 2 was determined to be 22.3 hours at pH 7.5 in 20 mM phosphate D\textsubscript{2}O buffer (Figure 3.14). The degradation was followed using the disappearance of the amidine peak of 2 at 8.03 ppm, which was accompanied by the complementary appearance of the amidine peak of 7 at 7.87 ppm (Figure 3.15).

\textbf{Figure 3.14} – Disappearance of 2, as followed by the amidine peak at 8.03 ppm.
Figure 3.15 – Appearance of 7, as followed by its amidine peak at 7.87 ppm
IV. SYNTHESIS AND CHARACTERIZATION OF 3
4.1 Synthesis and Characterization of 3 – Methods

The synthesis of 3 was analogous to that of 2. It required creation of Boc-protected D-alanyl-D-thiolactic acid (Scheme 3.1), followed by cleavage of the Boc protecting group (Scheme 3.2), and then formation of the amidine bond (Scheme 3.4).

![Scheme 4.1 – Formation of Boc-D-alanyl-D-thiolactic acid.]

**Synthesis of Boc-D-Alanyl-D-Thiolactic Acid**

Boc-D-alanine (2.8 g, 15 mmol), and CDI (2.4 g, 15 mmol) were dissolved in 90 mL of dry THF and allowed to stir on ice for 1 hour. At this point, D-thiolactic acid (1.45 mL, 16 mmol) was dissolved in 10 mL dry THF and added to the above solution. The reaction mixture was allowed to stir for 4 days at 5°C. The THF was removed on a rotary evaporator and the resulting residue was taken up in 75 mL of ethyl acetate. The solution was washed twice with 100 mL 10% citric acid solution, and twice with 100 mL water. The solution was dried over MgSO₄, filtered through a gravity filter. The ethyl acetate was removed on a rotary evaporator and the product was further dried on a vacuum pump overnight. The product solidified on drying, mostly white, but retaining a slight green hue. This crude product weighed 3.10 g (76% yield). The product recrystallized readily from diisopropyl ether, affording 1.02 g (25% yield) of colorless crystals. ¹H-NMR confirmed the purity of the crystals. ¹H-
NMR (CDCl₃): δ 4.93 (1H, d, J = 8.7 Hz, NH), 4.43 (1H, quintet, J = 7.5 Hz, CH), 4.19 (1H, q, J = 7.5 Hz, CH), 1.55 (3H, m, J = 6.9 Hz, CH₃), 1.46 (9H, s, Boc), 1.41 (3H, d, J = 5.1 Hz, CH₃), with the chloroform peak as the reference at 7.27 ppm (Figure 4.1). The melting point of the Boc-D-alanyl-D-thiolactic acid crystals was sharp at 110 °C.

Synthesis of 3: First Attempt

The deprotection of Boc-D-alanyl-D-thiolactic acid (100 mg, 0.4 mmol), was achieved as described above in the synthesis of 2, and is shown as Scheme 4.2.

Scheme 4.2 – Boc cleavage of Boc-D-alanyl-D-thiolactic acid.

The deprotected product from above was taken up in methanol (1.2 mL). Tertiary amine resin beads (230 mg, 0.9 mmol equivalent at 3.9 mmol/g) and hexamethyleneimine-carboxaldehyde dimethyl acetal (87 μL, 0.5 mmol) were then added, and the reaction mixture was allowed to stir at 5°C for 48 hours. The mixture was then filtered in a sintered glass funnel to remove the tertiary amine resin beads, and the beads were washed with 5 mL more methanol. The combined filtrate was dried on a rotary evaporator and dried further on a vacuum pump overnight. The crude product weighed 132 mg.
The crude product was taken up in 4.4 mL of water to create a 30 mg/mL solution, which was subsequently filtered by syringe filter, and run through the HPLC using the same gradient as was used above for compound 1 (Section 2.2). The flow rate was 3.3 mL/minute, 150 μL of sample was injected and absorbance was monitored at 240 nm. One major potential product peak appeared in the elution profile around 21.5 minutes. The peak was collected and lyophilized, but its $^1$H-NMR spectrum showed that the required product had methanolyzed, yielding 6. This result was confirmed by comparison with the products of the first and second synthesis attempts of 2, which also methanolyzed, and had the same HPLC peak retention time and $^1$H-NMR spectra.

Scheme 4.3 – Unsuccessful first synthesis of 3 – product methanolyzed.
Synthesis of 3: Second Attempt

The deprotection of the Boc-D-alanyl-D-thiolactic acid (100 mg, 0.4 mmol), was achieved as described above, yielding D-alanyl-D-thiolactic acid as a colorless glass, which was taken up in isopropanol (1 mL) and tertiary amine resin beads (92 mg, 0.4 mmol equivalent at 3.9 mmol/g) were added to the mixture. The reaction vessel was capped with a rubber septum and placed in a dry ice/acetone bath at -78 °C. Finally, hexamethyleneiminecarboxaldehyde dimethyl acetal (67 µL, 0.4 mmol) was added, and the reaction mixture was allowed to stir for 6 hours at -78 °C. The mixture was then allowed to come to room temperature, quickly filtered through a sintered glass funnel to remove the tertiary amine resin beads, and the beads were washed with 5 mL isopropanol. The combined filtrate was dried on a rotary evaporator and dried further on a vacuum pump overnight. The resultant glass, crude 3, weighed 189 mg.

The crude product mixture was taken up in 6.3 mL water to create a 30 mg/mL solution, and was run through the HPLC. The elution solvent gradient ran from 98% water to 84% water over 50 minutes. Sample injections and absorbance monitoring were as above. One major potential product peak appeared in the elution profile between 35 and 36 minutes (Figure 4.2). This peak was collected and freeze-dried, and its 1H-NMR spectrum was taken in 20 mM phosphate D2O buffer, pH 7.5: δ 8.02 (1H, s, amidine CH), 4.48 (1H, q, J = 7.2 Hz, methine CH), 4.00 (1H, q, J = 7.5 Hz, methine CH), 3.70 (2H, t, J = 6.0 Hz, NCH2), δ3.56 (2H, m, J = 6.3 Hz, NCH2), 1.84 (4H, br m, 2 ring CH2s), 1.63 (4H, br m, 2 ring CH2s), 1.54 (3H, d, J = 7.2 Hz,
ala methyl CH\textsubscript{3}), 1.46 (3H, d, J = 7.5 Hz, ala methyl CH\textsubscript{3}) with the DSS as the reference at 0.00 ppm (Figure 4.3). The spectrum contained all the necessary product peaks to be 3. Product identity was confirmed by mass spectrometry: m/z = 287.2 (Figure 4.4).

To prepare larger amounts of pure 3, the remaining crude 30 mg/mL solution was freeze-dried and made into a 60 mg/mL solution. In all, approximately 120 mg of crude 3 was run through the HPLC. Upon pooling and freeze-drying all the collected fractions containing 3, 11.6 mg of product were recovered.

Scheme 4.4 – Successful synthesis of 3.
Synthesis of 3: Third Attempt

In order to prepare more pure 3, the reactions of the second synthesis were repeated exactly, but with the amounts of all reactants doubled. The crude product amounted to 324 mg, which was dissolved in 5.40 mL water to make a 60 mg/mL solution. In all, approximately 190 mg of crude 3 was run through the HPLC. The elution profile was the same as in the previous synthesis. All compound 3 fractions were collected, pooled and lyophilized. The dissolved product was syringe-filtered before final lyophilization in a pre-weighed vial. Of the 190 mg of crude product purified, 46.2 mg of pure 3 were recovered. This represents a recovery rate of 24%, and indicates that for each 150 μL injection at 60 mg/ml, approximately 2.2 mg pure 3 can be recovered. This large amount of product was again confirmed to be 3, intact and pure by \(^1\)H-NMR. The product was a hard, sticky, peach-colored glass.

Aqueous Degradation of 3, Followed By \(^1\)H-NMR Spectroscopy

Approximately 2 mg of 3 were dissolved in a 20 mM phosphate D\(_2\)O buffer, pH 7.5 containing DSS as the reference at 0 ppm. Degradation was followed by \(^1\)H-NMR over 10 days (Figures 4.6 – 4.9). The fully degraded compound 3 \(^1\)H-NMR spectrum in 20 mM phosphate D\(_2\)O buffer, pH 7.5: δ 7.86 (1H, s, amidine CH), 4.13 (1H, q, J = 7.2 Hz, methine CH), 3.65 (2H, t, J = 5.7 Hz, NCH\(_2\)), 3.54 (1H, q, J = 7.2 Hz, free thiolactic acid methine CH), 3.50 (2H, t, J = 5.6 Hz, NCH\(_2\)), 1.81 (4H, br s, 2 ring CH\(_2\)s), 1.60 (4H, br s, 2 ring CH\(_2\)s), 1.48 (3H, d, J = 6.9 Hz, CH\(_3\)), 1.45 (3H, d, J = 7.2 Hz, free thiolactic acid CH\(_3\)), 1.43 (<1H, d, J = 7.5 Hz, unknown, could be thiolactic acid disulfide) with DSS as the reference at 0.00 ppm (Figure 4.5).
4.2 Synthesis and Characterization of 3 – Results

Compound 3 was prepared as shown in Scheme 4.5. The synthesis first required creation of the Boc-protected D-alanyl-D-thiolactic acid from Boc-D-alanine and D-thiolactic acid. After deprotection of the Boc group by TFA, the reactive amine was condensed with hexamethyleneiminecarboxaldehyde dimethyl acetal to afford 3.

Scheme 4.5 – Successful synthesis of 3.

Synthesis of Boc-D-Alanyl-D-Thiolactic Acid

The synthesis of the Boc-D-alanyl-D-thiolactic acid was successful. The yield of recrystallized material was 25%. The crystals were a fine, white powder. $^1$H-NMR confirmed the identity and purity of the crystals (Figure 4.1).
Figure 4.1 – $^1$H-NMR spectrum of Boc-D-alanyl-D-thiolactic acid in CDCl$_3$. 
Compound 3 is Susceptible to Methanolysis

The syntheses of 2 and 3 were run in parallel, and so methanol was used as the solvent in the first synthesis of 3. It was soon discovered that the thiolester bond in both compounds was readily methanolyzed, yielding compound 6. The methanolysis products from 2 and 3 had identical HPLC retention times, $^1$H-NMR spectra and masses. For the full discussion, refer to Section 3.2. The solvent was changed to isopropanol for the subsequent syntheses, which were successful.

Successful Synthesis of 3

As was seen in the synthesis of 2, the reaction was successful when run at -78 °C in isopropanol. The elution time of 3 from the HPLC, using the gradient above, was between 35 and 36 minutes (Figure 4.2). The identity of 3 was confirmed by $^1$H-NMR (Figure 4.3) and by mass spectrometry: m/z = 287.2 (Figure 4.4).
Figure 4.2 – HPLC elution profile of crude 3 from the third synthesis attempt. Compound 3 eluted around 36 minutes.

Figure 4.3 – $^1$H-NMR of 3 in 20 mM phosphate D$_2$O buffer, pH 7.5.
Figure 4.4 – Mass spectrum of 3.

Aqueous Degradation of 3, Followed by $^1$H-NMR Spectroscopy

The NMR spectrum of fully degraded compound 3 is shown below as Figure 4.5. The aqueous degradation of 3 was similar to that of compound 2, and it appeared that degradation consisted only of hydrolysis of the thiolester bond. Free thiolactic acid appeared to be released during the degradation, and the amidine bond remained intact because no free hexamethyleneimine was observed. The expected degradation is shown as Scheme 4.6.
Figure 4.5 – $^1$H-NMR of 3, completely hydrolyzed after 223 hours in 20 mM phosphate D$_2$O buffer, pH 7.5.
Scheme 4.6 – Hydrolysis of 3 in phosphate buffer, pH 7.5, yields thiolactic acid and compound 7.

To confirm the identity of the degradation products, the NMR spectrum of thiolactic acid was taken in 20 mM phosphate D$_2$O buffer, pH 7.5 and compared to the spectrum of fully degraded 3. The spectrum of the other degradation product, compound 7, was obtained above (Section 3.2). The comparison of all three spectra is shown as Figure 4.6. Peaks in the NMR spectrum of the fully degraded compound 3 are consistent with those of the amidino-alanine 7 and free thiolactic acid, confirming the validity of Scheme 4.6. Of note, the chemical shift of the free thiolactic acid methine and methyl peaks moved increasingly downfield as the amount of free
thiolactic acid increased over time, which is most likely an effect of the decreasing pH as more thiolactic acid was released during hydrolysis. The chemical shifts of the free thiolactic acid control were even further downfield because the sample concentration was greater than that in the Target 3 tube, and thus the pH was lower.

**Figure 4.6** – $^1$H-NMR of thioglycolic acid (top), compound 7 (middle) and fully degraded 3 (bottom), all in 20 mM phosphate D$_2$O buffer, pH 7.5.
The changes in the NMR spectrum of 3 over time are shown in Figure 4.7. The half life of 3 was determined to be 45.7 hours at pH 7.5 in 20 mM phosphate buffer (Figure 4.8). The degradation was followed using the disappearance of the amidine peak of 3 at 8.02 ppm, which was accompanied by the complementary appearance of the amidine peak of 7 at 7.87 ppm (Figure 4.9). As with compound 2, the amidine bond remains intact throughout the degradation to 7, which is very stable in aqueous solution at pH 7.5. A possible cause for this difference in activity from compound 1 will be discussed in Section 7.

**Figure 4.7** – $^1$H-NMR of 3 at 0 (top), 53 (middle) and 224 hours (bottom) in 20 mM phosphate D$_2$O buffer, pH 7.5.
Figure 4.8 – Disappearance of 3, as followed by the amidine peak at 8.02 ppm.
Figure 4.9 – Appearance of compound 7, as followed by the growth of its amidine peak at 7.87 ppm.
V. ENZYME KINETICS
5.1 Enzyme Kinetics – Methods

E. coli PBP2 Kinetics

The stock enzyme solution, a generous gift from Dr. H. Adachi, contained 20.3 μM PBP2. The buffer used for all enzyme kinetics studies was 50 mM phosphate, 0.5 M NaCl, pH 7.0. The studies were carried out at 37 °C.

Positive and negative controls as well as competition experiments used 6-β-bromopenicillanic acid (bromopenicillin) as a chromogenic inhibitor, and were monitored spectrophotometrically at 325 nm. The concentration of bromopenicillin was 100 μM in all tests. Enzyme concentration was 2.03 μM in all control and competition tests (10μL enzyme/100 μL for most runs). Positive controls contained only enzyme and bromopenicillin. Negative controls were performed with mecillinam (100 μM), which was incubated with enzyme for 10 minutes at 5 °C, followed by addition of bromopenicillin and monitored at 325 nm. In the competition experiments, substrates 1A, 1B, 2 and 3, at 1 mM concentration, were incubated with PBP2 for 0, 30 and 60 minutes, followed by addition of bromopenicillin. In the direct competition experiment (incubation time of 0) enzyme was added last to ensure true competition.

To monitor the PBP2 reaction with 1A and 1B as substrates, the absorption of the amide bond was followed directly at 230 nm. With 2 and 3, the thiolester absorption was monitored at 240 nm. Enzyme concentration was 0.4 μM and compound concentration was 100 μM. Experiments were also performed on 2 and 3 using 4,4-dipyridyl dithiol as a reporter for free thiol release. In this case, the same
buffer as above was employed, but containing 1 mM 4,4-dipyridyl dithiol. The reaction was monitored at 340 nm. PBP2 concentration was 2 μM, and compound concentration was 100 μM.

*Enterobacter cloacae* P99 β-Lactamase Kinetics

Positive and negative controls and direct monitoring experiments were repeated as above, but using the P99 β-Lactamase.
5.2 Enzyme Kinetics - Results

*E. coli* PBP2 Kinetics

The *E. coli* PBP2 sample available was a solublized construct created by removing the putative membrane anchor hydrophobic region at the N-terminal end of the protein (31 amino acids, residues 15-45) by site-directed mutagenesis. Dr. H. Adachi et. al. had shown that this construct retains \[^{[3]}H\]-benzylpenicillin-binding capability.\(^{27}\)

First, a control experiment was conducted to ensure the enzyme sample was active in some spectrophotometrically-measurable way. This positive control was performed with bromopenicillin, which has previously been shown to react with the solubilized construct of PBP2.\(^{14,28,29}\) Bromopenicillin reacts with the enzyme to yield a chromophore with an absorption maximum at 325 nm (\(\Delta \varepsilon_{325} \approx 12,000 \text{ cm}^{-1}\text{M}^{-1}\)).\(^{30,31}\) The chromophore formation reaction of bromopenicillin is shown as Scheme 5.1.\(^{32}\) Bromopenicillin did, in fact, react with PBP2 yielding the expected chromophore. The enzyme, however, appeared to vary considerably in its apparent concentration. Its effective concentration consistently seemed to increase over the course of the first several hours following thawing from the -70 °C freezer. Over the course of days to weeks, through several freeze-thaw cycles, the enzyme gradually lost nearly all of its activity. This loss in activity may be due to the enzyme aggregating as, over time, a small pellet of precipitate became visible upon centrifugation of the enzyme sample. Centrifugation of the fresh enzyme sample produced no visible pellet.
Because the mecillinam side chain is presumably the source of specificity for *E. coli* PBP2, and the focus of this thesis, a negative control was performed to ensure that mecillinam does, in fact, react with the enzyme. Mecillinam (100 μM) was incubated with PBP2 for 10 minutes at 5 °C, followed by addition of bromopenicillin. The reaction of bromopenicillin was completely inhibited by mecillinam (Figure 5.1). These control experiments provided evidence that the PBP2 sample provided by Dr. Adachi is active and reacts with at least bromopenicillin and mecillinam.
Figure 5.1 – Bromopenicillin (100 μM) and mecillinam react with *E. coli* PBP2 (4 μM). Bromopenicillin control, red circles; 10 minute pre-incubation with mecillinam (100 μM), blue diamonds.

To determine rate constants for the PBP2 reaction with compounds 1A and 1B, the absorption of the amide bond was followed directly at 230 nm. The absorbance was expected to decrease as the enzyme hydrolzed the substrates amide bond should be cleaved by the enzyme, and absorbance would decrease dramatically if the bond were being hydrolyzed.33, 34 However, only a slight difference was seen
between control (substrate hydrolysis alone) and enzyme-containing experiments (Figure 5.2). The best results obtained from the direct amide bond observation experiments yielded $k_{\text{cat}}/K_m$ values of $6 \text{ s}^{-1}\text{M}^{-1}$ and $20 \text{ s}^{-1}\text{M}^{-1}$ for compounds **1A** and **1B**, respectively. Similarly, PBP2 exhibited only very little reactivity with **2** or **3** when the thiolester absorption at 240 nm was monitored (Figure 5.3). The best results obtained from the direct thiolester bond observation experiments yielded $k_{\text{cat}}/K_m$ values of $6 \text{ s}^{-1}\text{M}^{-1}$ and $17 \text{ s}^{-1}\text{M}^{-1}$ for compounds **2** and **3**, respectively. None of the four compounds, therefore, appeared to be good substrates of PBP2.

*Figure 5.2 – Direct observation of compound **1A** at 230 nm. **1A** (100 μM) alone, red circles; **1A** (100 μM) with PBP2 (0.4 μM), blue squares.*
Figure 5.3 – Direct observation of compound 3 at 240 nm. 3 (100 μM) alone, red circles; 3 (100 μM) with PBP2 (0.4 μM), blue squares.

To further explore the possibility that 2 and 3 were substrates, assays were also performed in a buffer containing 4,4-dipyridyl dithiol, which reacts with free thiol groups to yield a chromophore with an absorption maximum at 340 nm (Δε$_{340}$ = 2,740 cm$^{-1}$M$^{-1}$)$^{35}$ (Scheme 5.2). The background hydrolysis rate of 4,4-dipyridyl dithiol with compounds 2 and 3 was significant, and the assays containing enzyme showed little difference from background (Figure 5.4). However, presence of the enzyme did cause a slight increase in the rate of chromophore formation, and using initial rate data, $k_{cat}/K_m$ values were calculated to be $3 ± 3$ s$^{-1}$M$^{-1}$ for compound 2.
(average of two runs) and $61 \pm 46 \text{s}^{-1}\text{M}^{-1}$ for compound 3 (average of 7 runs). The highest $k_{cat}/K_m$ values obtained in these experiments were $6 \text{s}^{-1}\text{M}^{-1}$ and $124 \text{s}^{-1}\text{M}^{-1}$ for 2 and 3, respectively. The rates are very small, and furthermore the reproducibility was very poor, as evidenced by the large standard deviations. This further confirmed that compounds 2 and 3 were poor substrates, at best, for *E. coli* PBP2.

![Scheme 5.2](image)

**Scheme 5.2** – Mechanism of chromophore formation from 4,4-dipyridyl dithiol$^{35}$
Figure 5.4 – 4,4-Dipyridyl dithiol (1 mM) assay with compound 3 (100 μM). The control is compound 3 alone, red circles; with PBP2 (2 μM), blue squares. These particular initial rates yield a calculated $k_{cat}/K_m$ of 124 s⁻¹M⁻¹ for 3.
Since the compounds were poor substrates, they were next evaluated as inhibitors. First, to determine if any of the compounds were fast, reversible inhibitors of PBP2, direct competition experiments against bromopenicillin were performed. The rate of the bromopenicillin reaction was only slightly decreased by the presence of compounds 1A, 1B, 2 and 3 and thus, the compounds are not strong fast-reversible inhibitors. The fit for the data in Figure 5.5 yielded a $K_i$ value of 800 μM for 3, assuming the inhibition to be competitive.

**Figure 5.5** – Competition of 3 and bromopenicillin for PBP2 (4 μM). In red: 3 (1 mM) and bromopenicillin (100 μM). In blue: bromopenicillin (100 μM) alone.
The final possibility explored was that the compounds were slow, tight-binding, irreversible inhibitors. If this were the case, the compounds would enter the PBP2 active site and remain bound there, covalently or non-covalently, which would block any subsequent bromopenicillin reaction. To investigate this, the enzyme was pre-incubated with the compounds (1 mM) for 0, 30 and 60 minutes, followed by addition of bromopenicillin. The absolute change in absorbance was not affected by incubation with any of the compounds 1A, 1B, 2 or 3 (Figure 5.6). Notably, the enzyme activity decreased precipitously on incubation at 37 °C.

**Figure 5.6** – Summary of tests for irreversible inhibition. None of the compounds was effective at blocking the bromopenicillin reaction. Note the loss of PBP2 activity after a short incubation period at 37 °C.
The compounds 1A, 1B, 2 and 3 were not good substrates, nor inhibitors of any kind of *E. coli* PBP2. In fact, the compounds were not definitively shown to bind or react with the enzyme in any way. With this discouraging result in mind, the final remaining issue to be explored was if the compounds react at all with any β-lactam-recognizing enzyme. The most promiscuous of the enzymes available in this laboratory is the *Enterobacter cloacae* P99 β-lactamase, and so it was the most likely of all enzymes to bind and react with the compounds 1A, 1B, 2 and 3.

As with PBP2, P99 was first shown to react with bromopenicillin and mecillinam. Bromopenicillin is a well-known β-lactamase inhibitor, and it does irreversibly inactivate the P99 enzyme. Addition of mecillinam did slow the reaction with bromopenicillin significantly, but did not block it completely.

### Table: Enterobacter cloacae P99 β-Lactamase Kinetics

<table>
<thead>
<tr>
<th>Compound</th>
<th>$k_2$ (s(^{-1})M(^{-1}))</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mecillinam</td>
<td>18,800</td>
<td>a</td>
</tr>
<tr>
<td>Benzylpenicillin</td>
<td>230</td>
<td>a</td>
</tr>
<tr>
<td>Bromopenicillin</td>
<td>35</td>
<td>b</td>
</tr>
<tr>
<td>1:A</td>
<td>≤ 6</td>
<td>b</td>
</tr>
<tr>
<td>1:B</td>
<td>≤ 20</td>
<td>b</td>
</tr>
<tr>
<td>2</td>
<td>≤ 6</td>
<td>b</td>
</tr>
<tr>
<td>3</td>
<td>≤ 130</td>
<td>b</td>
</tr>
</tbody>
</table>

*Figure 5.7 – Summary of All *E. coli* PBP2 Reaction Rates.*

a, calculated from data in Spratt, 1977; b, this work.

*Enterobacter cloacae* P99 β-Lactamase Kinetics

The compounds 1A, 1B, 2 and 3 were not good substrates, nor inhibitors of any kind of *E. coli* PBP2. In fact, the compounds were not definitively shown to bind or react with the enzyme in any way. With this discouraging result in mind, the final remaining issue to be explored was if the compounds react at all with any β-lactam-recognizing enzyme. The most promiscuous of the enzymes available in this laboratory is the *Enterobacter cloacae* P99 β-lactamase, and so it was the most likely of all enzymes to bind and react with the compounds 1A, 1B, 2 and 3.

As with PBP2, P99 was first shown to react with bromopenicillin and mecillinam. Bromopenicillin is a well-known β-lactamase inhibitor, and it does irreversibly inactivate the P99 enzyme. Addition of mecillinam did slow the reaction with bromopenicillin significantly, but did not block it completely.
Presumably, the mecillinam was either competing with bromopenicillin for the P99 active site in a fast, reversible manner, or the enzyme was reacting with and hydrolyzing mecillinam, as a β-lactamase would be expected to do. However, even this nonspecific enzyme failed to react with compound 1A, 1B, 2 or 3 at any reasonable rate.
VI. *IN SILICO* INVESTIGATIONS OF *E. COLI* PBP2
6.1 Molecular Modeling

Molecular Modeling Methods

Although there is no crystal structure of *E. coli* PBP2, there are crystal structures of closely related PBPs, for example, *Streptococcus pneumoniae* PBP2x (20% sequence identity, E-value $7 \times 10^{-16}$ as determined by a simple BLAST query at SwissProt, Section 6.3) which belongs to subclass B4 under the classification scheme of Ghuysen. Luckily, a literature search produced an article written in Japanese which seemed to discuss a computer model of *E. coli* PBP2. The lead author of the paper, Dr. Masaji Ishiguro of the Suntory Institute for Bioorganic Research, was kind enough to send us the model his group had created. The model was generated from the crystal structure of *Streptococcus pneumoniae* PBP2x (PDB accession code 1QME), by replacing the amino acid side chains to be those of *E. coli* PBP2 and allowing the structure to energy minimize, creating a homology model of PBP2.

Computations were run on an sgi octane2 workstation with the INSIGHT II 2005 suite of molecular modeling programs. The computations were carried out using the homology model from Dr. Ishiguro. With the aid of the molecule builder module of INSIGHT II, a tetrahedral intermediate of compound 1B was created in the active site of the PBP2 model, bound to the reactive serine, Ser 73. MNDO charges were employed for the ligand, calculated in INSIGHT II 2000, while protein charges were assigned by INSIGHT II 2005. The ligand was initially oriented with the oxyanion in the oxyanion hole and the amidine bond of the side chain in close proximity to Asp 132. Water molecules were added to the assembly as a sphere of 15 Å radius centered
at the Ser 73 Oγ. The Discover program was used for energy minimization and molecular dynamics simulations on the hydrated protein-ligand assembly. Energy minimization was allowed to run for 200 steepest descents, followed by molecular dynamics simulations, which were run for 20,000 steps at 300 K. The history file of this run contained snapshots at every 200th step for a total of approximately 100 snapshots. Several typical snapshots of the history file were selected and each was allowed to further energy minimize an additional 1000 steepest descents in the CV force field, followed by 2000 conjugate gradient steps.

A construct of meso-aminopimelyl-D-alanyl-D-alanine was also created and attached to the reactive serine, and the resultant assembly was treated as described above.

Molecular Modeling Results

Despite the discouraging enzyme kinetics results with compounds 1, 2 and 3, mecillinam does react with PBP2 and there is no obvious reason why the mecillinam side chain should fit in the PBP2 active site when attached to the penicillin but not when it is attached to the dipeptide. Computer modeling was employed to explore the nature of the amidine-PBP2 interaction.

First, the homology model provided by Dr. Ishiguro was compared to that of the parent crystal structure of S. pneumoniae PBP2x (Figure 6.1). The two active sites are nearly identical, save the mutation of Asn 397 in the PBP2x crystal structure to an Asp (position 132) in the E. coli PBP2 model. This is the Asn of the conserved SXN
motif, mutated to an SXD motif in *E. coli* PBP2, which is briefly described in the introduction.

Figure 6.1 – Comparison of the *S. pneumoniae* PBP2x crystal structure with the *E. coli* PBP2 homology model provided by Dr. Ishiguro. Note the Asn 397 of the SXN motif in PBP2x is replaced by the highly unusual Asp 132 mutation in *E. coli* PBP2.

Confident that Dr. Ishiguro’s homology model was acceptable, compound 1B was docked into the active site, covalently bound to the reactive serine, Ser 73, as a tetrahedral intermediate, and the assembly was energy minimized and the dynamics performed. In the snapshots of the PBP2 model with compound 1B (Figures 6.2 and 6.3), the central carbon atom of the amidine bond is positioned 3.3 Å away from the nearest oxygen of Asp 132. The amidine is also 3.5 Å away from the backbone carbonyl of Thr 290 on the opposite side of the active site. The oxyanion sits nicely in the oxyanion hole, and the terminal D-alanine carboxylate is within hydrogen
bonding distance to Lys 287 and the side chain hydroxyl of Thr 290. Interestingly, Trp 113 has flipped down on top of the active site during the course of the dynamics simulations (Figures 6.2 and 6.3).

Figure 6.2 – Snapshot of the *E. coli* PBP2 homology model with compound 1B bound at the reactive serine. The central carbon of the positively charged amidine is 3.3 Å away from the nearest oxygen of the negatively charged Asp 132.
Figure 6.3 – Side view of the PBP2 active site with compound 1B bound. This view, looking down the amidine bond, clearly shows that Trp 113 has flipped down directly on top of the amidine side chain, effectively blocking access to the active site.

A construct of the peptidoglycan-mimetic *E. coli* PBP2 substrate, aminopimelyl-D-alanyl-D-alanine, was also incorporated into the model and the assembly treated as above. In the snapshots of the PBP2 model with this substrate molecule (Figure 6.4), the oxyanion sits nicely in the oxyanion hole, and the terminal D-alanine carboxylate is within hydrogen bonding distance to Lys 287 and the side chain hydroxyl of Thr 290, just as was seen in the 1B assembly structures. The
primary ammonium ion of the aminopimelyl side chain is 2.8 Å away from the nearest oxygen of Asp 132. The carboxylate of the aminopimelyl is 3.7 Å away from Lys 115 and 3.5 Å away from Arg 310.

**Figure 6.4** - Snapshot of the *E. coli* PBP2 homology model with aminopimelyl-D-alanyl-D-alanine bound at the reactive serine. The terminus of the aminopimelyl is in close contact with Asp 132, Lys115 and Arg 310.
6.2 PBP2 Homology Search and Sequence Alignment

Homology Search and Sequence Alignment Methods

The *E. coli* PBP2 amino acid sequence (primary accession number P0AD65) was retrieved from the SwissProt Protein Knowledgebase. The PBP2 sequence was then submitted for a BLAST query against the SwissProt/TrEMBL Knowledgebase. The search was limited to the best 250 scoring sequences. Of these sequences, several of those containing the SXD motif were selected and aligned in the ClustalW program, version 1.81. This multiple sequence alignment is shown as Figure 6.5.

Homology Search and Sequence Alignment Results

Hundreds of species of bacteria carrying PBPs with the SXD motif were found in the homology search. Several are included in the sequence alignment shown in Figure 6.5. They all appear to be PBP2s and are exclusively from Gram-negative bacterial species. Several other Gram-negative genera, including *Bordatella*, *Enterobacter*, *Klebsiella*, and *Pseudomonas* were also found to contain species with PBPs exhibiting the SXD motif, but are not included in Figure 6.5.
<table>
<thead>
<tr>
<th>Species</th>
<th>Sequence Alignment</th>
</tr>
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<tbody>
<tr>
<td>Candidatus Blochmannia</td>
<td>WKKWGHGELNITKALEES\textbf{SADTFFFYQIAYKM3IDNLSBMWKNKFYGKYGRTGI}</td>
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<tr>
<td>Blochmannia floridanus</td>
<td>WKKWGHGELNITKALEES\textbf{SADTFFFYQIAYKM3IDNLSBMWMTKFGYKYGRTGI}</td>
</tr>
<tr>
<td>Escherichia coli K12</td>
<td>WKKWGHGRLNVTRESLES\textbf{SADTFFFYQVAYDM3IDLSEWMKGFKFGYHYRTGI}</td>
</tr>
<tr>
<td>PBP2 – E. Coli</td>
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</tr>
<tr>
<td>Shigella dysenteriae</td>
<td>WKKWGHGRLNVTRESLES\textbf{SADTFFFYQVAYDM3IDLSEWMKGFKFGYGSLRTGI}</td>
</tr>
<tr>
<td>Salmonella enterica</td>
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<td>Enterobacter sakazakii</td>
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<tr>
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<td>Yersinia pestis</td>
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<tr>
<td>Sodalis glossinidius</td>
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<tr>
<td>Baumannia cicadellinicola</td>
<td>WKKWGHGRLNVTRESLES\textbf{SADTFFFYQVAYDM3IDLSEWMKGFKFGYHYRTGI}</td>
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<tr>
<td>Salmonella enterica</td>
<td>WKKWGHGMLDVTKALEES\textbf{SADTFFFYQVAYKM3IDRIDLMSQFGYKGTIGI}</td>
</tr>
<tr>
<td>Mannheimia succiniciproducens</td>
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<tr>
<td>Pasteurella multocida subsp</td>
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</tr>
<tr>
<td>Haemophilus ducreyi</td>
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<tr>
<td>Photobacterium profundum</td>
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<tr>
<td>Vibrio vulnificus</td>
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</tr>
<tr>
<td>Vibrio cholerae</td>
<td>WKKWGHGMLDVTKALEES\textbf{SADTFFFYQVAYDM3IDLSEWMKGFKFGYHYRTGI}</td>
</tr>
</tbody>
</table>

**Figure 6.5** – Sequence alignment of several PBPs which all contain the SXD motif (underlined). Blue indicates a residue which is fully conserved, while green indicates residues which are functionally conserved. Created with ClustalW v1.81.
VII. DISCUSSION
**Syntheses and Characterization**

Compounds 1, 2 and 3 were successfully synthesized, purified and characterized. The synthesis and purification of 1, however, was complicated by the epimerization of the N-terminal alanine α-carbon, which occurred during the synthesis (Section 2.3). A stereospecific synthesis of 1 was eventually achieved by running the reaction at -78 °C (Section 2.3). This reaction condition was also successful in the stereospecific synthesis of 2 and 3, but the solvent was changed to isopropanol to prevent methanolysis of the thiolester (Sections 3.2 and 4.2). The stability of the compounds in buffered aqueous solution was also studied and each compound was determined to be sufficiently robust to allow for enzyme kinetics studies (Sections 2.3, 3.2 and 4.2). The degradation of 1 yielded hexamethyleneimine and a diastereomeric mixture of N-formyl-alanyl-D-alanine (Schemes 2.5 and 2.6). The degradation of compounds 2 and 3, however, simply involved hydrolysis of the thiolester, yielding the amidino-D-alanine 7 and thioglycolic acid or D-thiolactic acid, respectively (Schemes 3.7 and 4.6).

**Mechanism of Epimerization**

The degradation of compound 1 was intriguingly different from that of compounds 2 and 3. Hydrolysis of compound 1 was accompanied by concurrent epimerization of the alanine adjacent to the amidine, while compounds 2 and 3 did not epimerize during thiolester hydrolysis. Furthermore, the amidine bond in compound 1 hydrolyzed readily, but did not in the degradation of 2 and 3. Also, the
amidino-D-alanine 7 seems to be infinitely stable in buffered aqueous solution. These observations are puzzling at first, but a closer examination of the proposed mechanism of hydrolysis for compound 1 (Schemes 2.5 and 2.6) may explain the source of this divergent behavior (Figure 7.1).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Can it form</th>
<th>Does it epimerize?</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A, 1B</td>
<td>Yes, 4 &amp; 5</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Prevented at -78°C</td>
</tr>
<tr>
<td>2, 3</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>6, 7</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>N-formyl-alanyl-alanine</td>
<td>Yes, 8:</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Prevented at 5°C</td>
</tr>
</tbody>
</table>

**Figure 7.1** – Comparison of all compounds and their ability to form the cyclized intermediate. The compounds which are able to form the intermediate have a nitrogen at position X and are also the compounds which epimerize.

Compound 1 can readily form the cyclized intermediate 4 because the nitrogen atom (in position X) of the amide bond to the terminal D-alanine has a lone electron pair which is available to form the iminium species. This cyclized intermediate 4 would make the methine acidic, facilitating epimerization. However,
the sulfur atom of the thiolester cannot donate a lone electron pair to the adjacent carbonyl because of the poor overlap of its lone electron pairs in the third energy level with the carbon valence shells in the second energy level. Thus, the thiolester compounds 2 and 3 would be unable to epimerize by this mechanism and accordingly, no epimerization is observed during their degradation. The oxygen atom of the ester bond in the methanolysis product 6 (Scheme 3.3) can, in theory, donate its lone electron pairs to the carbonyl, but oxygen is more electronegative than nitrogen, and thus epimerization of the methyl ester 6 by way of the cyclized intermediate would be expected to occur more slowly than with the amide compounds, if at all. Indeed, no epimerization is observed in 6 before or during the hydrolysis of the methyl ester. Compound 7 also has an oxygen in this position, but at pH 7.5 it would be deprotonated and its negative charge would be resonance-stabilized across the three atoms of the carboxylate, which would be highly unlikely to attack the amidine. The only other compounds studied in this thesis with a nitrogen in position X were the N-formyl dipeptides. These do, indeed, epimerize if their synthesis is performed at room temperature. However, unlike with compound 1, the resultant cyclized intermediate 8 (Figure 7.1) has no leaving group and thus water cannot attack, and the structure eventually reopens to epimerized starting material rather than hydrolyzing.

Epimerization of the N-formyl dipeptides is prevented if the synthesis is performed at 5 °C. Similarly, the synthesis of compound 1 must be performed at -78 °C in order to prevent epimerization. Even this difference in susceptibility to epimerization between compound 1 and the N-formyl dipeptides can be explained by
the cyclic intermediate mechanism. Because the amidine is positively charged, it is presumably more electrophilic than the carbon of the aldehyde group in the N-formyl dipeptides, suggesting that a lower temperature would be required to prevent attack of the nucleophilic amide and the resultant epimerization. This proposed mechanism provides a simple explanation of the behavior of all compounds synthesized and studied in this thesis. An investigation of the degradation of 1 in $[^{18}\text{O}]$-labeled D$_2$O may be able to confirm the validity of the mechanism.

**Enzyme Kinetics**

The water-soluble *E. coli* PBP2 construct provided by Dr. Adachi was active *in vitro*, reacting with both bromopenicillin and mecillinam (Section 5.2). However, the enzyme sample was quite variable in its apparent concentration after thawing from frozen solution, and gradually lost nearly all activity on repeated freezing and thawing, presumably because the enzyme precipitated (Section 5.2). The enzyme was also very unstable, losing more than half of its activity after 30 minutes of incubation at 37 °C (Figure 5.6). The cause of this changeable performance is unclear, but the behavior may have contributed to the poor reproducibility observed in some of the kinetics assays.

Compounds 1, 2 and 3 all failed to react with the enzyme at any significant rate (Section 5.2). The thiodepsipeptides were expected to be better substrates than the peptide compounds, and indeed, compound 3 was slightly more promising than the other substrates with a $k_{\text{cat}}/K_m$ of $\leq 130$ $\text{s}^{-1}\text{M}^{-1}$, but the reproducibility of this value was poor (Section 5.2). The compounds 1, 2 and 3 also failed to behave as either fast,
reversible or slow, tight-binding irreversible inhibitors of *E. coli* PBP2 (Section 5.2). These results should not be entirely surprising, given the lack of activity of many HMW PBPs against most substrates *in vitro.*\(^3\)-\(^5\), \(^15\) Furthermore, the low substrate \(k_{cat}/K_m\) values reported in this thesis are nearly identical to those obtained with a water-soluble construct of *E. coli* PBP3 which has been shown to catalyze the transpeptidation reaction with thiolester compounds similar to 2 and 3.\(^6\) It remains unclear why *E. coli* PBP2 would bind and react with mecillinam, but not with peptide or thiodepsipeptide substrates bearing the mecillinam side chain.

**Molecular Modeling**

A sequence alignment of the active site residues of numerous PBPs from a variety of species shows that *E. coli* PBP2 contains a mutation in the SXN motif, which is conserved in nearly all PBPs, to a unique SXD motif in PBP2 (Figure 1.9). Numerous site-directed mutagenesis studies have implied a substrate recognition role for the SXN motif (discussed below), and numerous crystal structures\(^2\), \(^40\), \(^41\) have demonstrated that the Asn of the SXN motif forms a hydrogen bond with the carbonyl of the amide linker of penicillin and cephalosporin side chains. It is intriguing to consider the coincidence that mecillinam is the only penicillin with the positively charged amidine side chain linker while *E. coli* PBP2 is the only PBP with a negatively charged Asp mutation in the conserved active site SXN motif, and that mecillinam is a potent inhibitor of only *E. coli* PBP2. It is possible that this mutated residue electrostatically interacts with the mecillinam side chain, thereby contributing
to the unique behavior exhibited by *E. coli* PBP2. Molecular dynamics simulations were the only tool readily available to investigate this hypothesis, and were performed with compound 1B docked in the PBP2 active site.

In the snapshots of the PBP2 model with compound 1B, the positively charged amidine side chain is within 3.3 Å of the negatively charged Asp 132 residue apparently forming an electrostatic interaction (Figure 6.2). This result suggests that the Asp-amidine electrostatic interaction also occurs with mecillinam itself *in vivo* (Figure 7.2). The Asn which occupies this position in all other PBPs is neutral and would be incapable of forming a strong electrostatic interaction with the positively charged amidine. Accordingly, no SXN-containing PBP has been demonstrated to react with mecillinam.

![Diagram](image)

**Figure 7.2** – Comparison of the possible modes of binding of mecillinam in the *E. coli* PBP2 active site with benzylpenicillin in the *S. pneumoniae* PBP2x active site.
It is also interesting to note that Trp 113 has flipped down on top of the active site during the course of the dynamics simulations, effectively blocking access to the active site (Figures 6.2 and 6.3). It is possible that this residue prevents the substrates 1, 2 and 3 from gaining access to the PBP2 active site, however, it is unclear how the Trp could selectively block substrates while failing to block the reaction with mecillinam or bromopenicillin. *In vivo*, this Trp residue must surely be pushed away from the active site, perhaps by the large, continuous peptidoglycan substrate, or by an allosteric interaction with either the partner protein RodA or with some other element of the local environment.

If Asp 132 is sufficient to decrease PBP2’s affinity for normal penicillins and cephalosporins, then it is possible that the natural substrate interacts differently with PBP2 than with other PBPs. To address this question, a portion of the natural *E. coli* substrate, aminopimelyl-D-alanyl-D-alanine, was modeled in the PBP2 active site. The snapshots of the dynamics show that Asp 132 could, indeed, interact with the positively charged terminal amino group of the pimelyl moiety of natural substrate. In fact, the natural substrate seems to fit quite nicely in the PBP2 active site, forming several hydrogen bonds and electrostatic interactions (Figure 6.4).

**PBP2 Homology Search and Multiple Sequence Alignment**

The PBP2 homology search and sequence alignment demonstrated that hundreds of species of bacteria carry PBPs with the SXD motif. Many of these bacterial species are clinically important, including species from the *Bordatella,*
Enterobacter, Haemophilus, Klebsiella, Pseudomonas, Salmonella, Serratia, Shigella, Vibrio, and Yersinia genera. Of these, mecillinam has been shown to be effective against species of the Bordatella, Enterobacter, Haemophilus, Klebsiella, Pseudomonas, Salmonella, Serratia, and Shigella genera as well as others, and is likely to be effective against many more. Every protein with the SXD sequence detected by the search belonged to a Gram-negative bacterial species. Importantly, no Gram-positive bacteria were found that contain PBPs with the SXD motif.

Mecillinam is far more effective against Gram-negative bacteria than Gram-positive bacteria. This behavior is highly unusual for penicillins, which are typically much more effective against Gram-positives, presumably because they lack the protective outer membrane. Mecillinam’s reversed specificity profile prompted first Blumberg and Strominger, and later Spratt and Pardee, to postulate that mecillinam acts in a highly specific manner on some target which is present in Gram-negative organisms but lacking in Gram-positive organisms. This hypothesis remains unproven and untested, however, the sequence alignments reported in this thesis suggest that the hypothesis of Blumberg and Strominger is, indeed true, and that the Gram-negative specific targets are the SXD-containing PBPs.

Mecillinam and β-lactamases

Mecillinam has long been known to be effective against Gram-negative bacteria, including many which produce β-lactamases. A recent article specifically addressed the comparative efficacy of mecillinam and other antibiotics against a
variety of β-lactamases. Mecillinam was found to be the most effective of the nine antibiotics tested, including clavulanic acid, against bacterial strains producing the class A β-lactamases TEM-1, TEM-3, SHV-3, and IRT-5, as well as the class C β-lactamase AmpC. All these β-lactamases contain the typical SXN motif at their active sites, and so it is perhaps not surprising that they fail to react with mecillinam just as is observed in SXN-containing PBPs. The class D enzyme, OXA-3, was the only one examined that could effectively hydrolyze mecillinam. OXA-3 and many other class D lactamases have a mutation in the SXN motif to SXV. The fact that OXA-3 contains a mutation in the same position of the SXN motif as that of E. coli PBP2, and that it also is able to react with mecillinam further suggests that the N-position of the SXN motif plays an important role in mecillinam binding.

Other SXN Mutants

A more focused investigation of the specific importance of the N-position of the SXN motif in the recognition of substrates would be to mutate the residue, excluding all other variables, and directly compare the activity of the mutant enzyme to that of the wild-type enzyme. Fortunately, several mutants of Asn the in the SXN motif have been created and studied in HMW and LMW PBPs as well as in some β-lactamases. In each case, the mutation caused a profound change in enzyme behavior, both in specificity profile for β-lactams and in the ability to turn over substrates (Figure 7.3). Interestingly, the behavior of all the SXN-mutant enzymes seems to be modified such that it becomes more comparable to the behavior of E. coli PBP2.
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>E. coli PEP3</th>
<th>E. coli PEP5</th>
<th>Actinomadura R39</th>
<th>Streptomyces R61</th>
<th>Streptomyces ribose G- β-lactamase</th>
<th>TEM β-lactamase</th>
<th>E. coli PEP2</th>
<th>Streptomyces K15</th>
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<tbody>
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<td>HMW</td>
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<td><strong>Mutation</strong></td>
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<td>SXS</td>
<td>SXS</td>
<td>SXS</td>
<td>SXS</td>
<td>SXD</td>
<td>SXD (WT)</td>
<td>SXC (WT)</td>
<td>SXS (WT)</td>
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<tr>
<td><strong>Effect on Reactivity with Benzylpenicillin</strong></td>
<td>Minimal</td>
<td>Loss</td>
<td>Minimal</td>
<td>Minimal</td>
<td>Moderate</td>
<td>230</td>
<td>3 × 10^5</td>
<td>3 × 10^6</td>
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<tr>
<td><strong>Effect on Reactivity with Cephalosporin</strong></td>
<td>Loss (a)</td>
<td>Loss (b)</td>
<td>Loss (c)</td>
<td>Minimal (c)</td>
<td>Loss (d)</td>
<td>Loss (b) &lt; 0.9 (a), (b)</td>
<td>2 (d)</td>
<td>7 × 10^3</td>
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<td></td>
<td>Loss (e)</td>
<td>Loss (f)</td>
<td>Loss (g)</td>
<td>Loss (f)</td>
<td>n/a</td>
<td>n/a</td>
<td>15 (f)</td>
<td>n/a</td>
<td></td>
</tr>
<tr>
<td><strong>Reactivity with substrates</strong></td>
<td>Loss (e)</td>
<td>Loss (f)</td>
<td>Loss (g)</td>
<td>Loss (f)</td>
<td>n/a</td>
<td>n/a</td>
<td>15 (f)</td>
<td>n/a</td>
<td></td>
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<td>59</td>
<td>7, This Thesis</td>
<td>52, 53, 54</td>
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**Figure 7.3** – Comparison of several SXN-mutant enzymes’ activities with benzylpenicillin, cephalosporins and substrates. Minimal = between 150% and 50% of WT activity; Moderate = 68% loss of WT activity; Loss = > 95% loss of WT activity. (a) cephalxin; (b) WT enzyme does not react with cephalosporins; (c) cephalosporin C and 7-APA; (d) cephalothin; (e) indicated by growth of pointed cell poles; (f) N-diacetyl-L-lys-D-ala-D-ala (g) N-acetyl-L-lys-D-ala-D-ala; (h) Compound 3; all numbers given are in s⁻¹M¹.
Mutation of the SXN motif to SXS has been studied in *E. coli* PBP3, *E. coli* PBP5, *Actinomadura* R39 and *Streptomyces* R61 DD-peptidases. In three out of four of these mutant enzymes the affinity for cephalosporins decreased to less than 5% of that exhibited by the wild type enzyme while having only a minimal effect of the enzymes’ reactivity with penicillins. Mutation of the Asn of the SXN motif also caused a decrease in the enzymes’ ability to process substrate in each PBP studied. The wild-type *Streptomyces* K15 PBP has a unique SXC motif, and it also exhibits a relatively normal reactivity with penicillins but a low reactivity with cephalosporins. The K15 enzyme turns over N-diacyetyl-L-lysyl-D-alanyl-D-alanine substrate even more slowly than the SXS mutants.

The SXN to SXS mutation has also been performed in β-lactamases. The mutation of SXN to SXS in *Streptomyces albus* G β-lactamase resulted in “spectacular modifications of the specificity profile of the enzyme.” The mutant enzyme had nearly unchanged penicillinase activity, but lost ≥ 99% of its cephalosporinase activity. The SXS motif even exists naturally in the *Bacillis cereus* β-lactamase III. This class A β-lactamase also exhibits a strong preference for penicillins over cephalosporins, with the $k_{cat}/K_m$ value of benzylpenicillin being approximately 500 times that of cephalexin. The class D β-lactamase OXA-3, discussed above, has an SXV motif, and it also exhibits high activity against penicillins but low activity against cephalosporins.

Apparently, only one mutant has been made with an SXD motif, which was in the TEM β-lactamase. Interestingly, the mutation decreased the activity of the β-
lactamase such that it was unable to confer resistance to antibiotics. Recall that mecillinam is resistant to hydrolysis by many \(\beta\)-lactamases, including TEM (discussed above). It is distinctly possible that the PBP2-like SXD motif of this mutated TEM would be sufficient to increase its hydrolytic activity against mecillinam, especially considering that the SXV motif of OXA-3 is sufficient to do so. This question could easily be answered and is worth investigating further.

It is interesting to note that \(E. \text{coli}\) PBP2, like the other SXN-mutant enzymes in Figure 7.3, also has a very low affinity for most cephalosporins and yet does react with benzylpenicillin.\(^7\) The mutation of the Asn, therefore, causes penicillin-interacting enzymes to discriminate between two compounds that are very similar in structure. One difference between penicillins and cephalosporins lies in the geometry of the lactam nitrogen, which is roughly trigonal pyramidal in penicillins, but planar in cephalosporins. In peptides, the analogous nitrogen is also held planar in the amide bond. Furthermore, despite the valiant efforts put forth in this thesis, PBP2 also has little to no activity on peptide substrates \textit{in vitro}. This coincidence does not explain the lack of PBP2 reactivity with the thiolester compounds, however, which need not be planar at this position. In any event, it is fascinating that mutation of the Asn of the SXN motif to either Asp, Cys or Ser causes a decreases in cephalosporin reactivity while having little effect on penicillin reactivity. Furthermore, these mutations cause this change in behavior in both HMW and LMW PBPs and even in \(\beta\)-lactamases. Clearly, the Asn of the SXN motif has a critical role in substrate recognition in PBPs and \(\beta\)-lactamases.
Mecillinam and *E. coli* PBP2

Mecillinam is exquisitely specific for *E. coli* PBP2, and it is the only antibiotic with the positively charged amidine linker, while PBP2 is the only PBP with a negatively charged Asp mutation in the N-position of the conserved active site SXN motif. Molecular modeling simulations suggest that the amidine could, indeed, form an electrostatic interaction with Asp 132. Furthermore, hundreds of species of Gram-negative bacteria carry SXD-containing PBPs, and mecillinam is effective only against Gram-negative bacteria. Mecillinam fails to react with SXN-containing PBPs, including most β-lactamases. However, OXA-3, with the SXV mutation, has been proven to react with mecillinam, and in general, all reported mutations of the N-position of the SXN motif have a profound effect on enzyme specificity and reactivity. Moreover, this effect tends to reflect the behavior exhibited by *E. coli* PBP2. In light of these facts, it is not at all radical or outrageous to suggest that the Asp of the SXD motif of *E. coli* PBP2 is solely responsible for the dramatic difference of its reactivity profile from that of other PBPs, and that an electrostatic interaction between the positively charged amidine and the negatively charged Asp is the reason that PBP2 binds and reacts with mecillinam in such a specific manner. It is, perhaps, far more surprising that the SXD mutation of *E. coli* PBP2, and its accompanying change in specificity, has been completely overlooked until now.
Conclusions and Future Work

The compounds synthesized in this study were poor substrates at best for *E. coli* PBP2. Indeed, the compounds were not definitively shown to interact with the enzyme in any way. All HMW PBPs exhibit little to no reactivity with substrates *in vitro*, but the low levels of activity observed here are particularly surprising. It would seem that incorporation of the amidine moiety alone is insufficient to increase the affinity of peptide or thiodepsipeptide substrates for *E. coli* PBP2. It remains unclear why *E. coli* PBP2 is able to bind and react with mecillinam, but not with substrates bearing the mecillinam side chain. However, the investigations discussed in this thesis provide significant insight into the nature of mecillinam’s specificity for *E. coli* PBP2. Site-directed mutagenesis studies would confirm the role of Asp132 proposed above.
VIII. REFERENCES


