Design and Synthesis of O-Aryloxycarbonyl Hydroxamate Inhibitors for Serine β-Lactamases

by

Lexie Malico
Class of 2016

A thesis submitted to the faculty of Wesleyan University in partial fulfillment of the requirements for the Degree of Bachelor of Arts with Departmental Honors in Chemistry

Middletown, Connecticut April, 2016
Acknowledgements

I would like to thank Professor Rex Pratt, first and foremost, for his encouragement and guidance during my time at Wesleyan University. You have been an extraordinarily patient mentor. I am incredibly grateful. This thesis would not have been possible without you. It has been a privilege and a pleasure to learn from you.

I would also like to thank the other members of Pratt Laboratory, both past and present: Venkatesh Nemmara, Ronak Tilvawala, Kinjal Dave, Angela Yoo, Sarah Newman, and Dr. Suara Adediran. Your assistance and guidance are so appreciated.

I would also like to thank all of the friends who have supported me through this process. Thank you all for putting up with me. I know that it has not always been easy, but I appreciate your support and encouragement beyond what I can express. Thank you to Jason Gibson, TJ Blackburn, Annee Barrett, Jacques Bazile, Sheri Reichelson, and Samantha Hellberg. You have all gone above and beyond what I could have ever asked for.

Lastly, thank you to my wonderful family. Your love and support has meant the world to me. Thank you, Mom, Lillian, Bob, Wendy, Molly, Ben, and Watson. You mean the world to me.
Abstract

β-Lactam antibiotics are commonly used in clinical practice to treat bacterial infections. Increasing bacterial resistance to β-lactam antibiotics is primarily attributed to the production of hydrolytic enzymes termed β-lactamases. Therefore, the development of novel β-lactamase inhibitors is critical. Recent investigations by Dr. Ronak Tilvawala in this laboratory into O-(phenoxy carbonyl)-N-[4-amino-4-carboxyl-1-butyl]oxycarbonyl]hydroxylamine (L2) revealed that the compound inhibits the class C serine β-lactamase of Enterobacter cloacae P99 by forming a hydroxamate acyl-enzyme complex, most likely stabilized by the Tyr 150, Ser 212, and Arg 204 residues in the Ω-loop of the active site. In the present research, several novel O-aryloxy carbonyl hydroxamate derivatives were designed and synthesized as potential P99 inhibitors in order to elucidate the importance of both the polar and nonpolar groups on the hydroxamic acid side chain of the inhibitor. Kinetic analysis indicated that O-(phenoxy carbonyl)-N-(1-butyloxycarbonyl]hydroxylamine (1), O-(phenoxy carbonyl)-N-[2-benzyl-1-ethyl] oxycarbonyl]hydroxylamine (2), O-(phenoxy carbonyl)-N-[4-carboxyl-1-butyl]oxycarbonyl] hydroxylamine (3), and O-(phenoxy carbonyl)-N-[4-amino-1-butyl]oxycarbonyl]hydroxylamine (4) were irreversible inhibitors of P99 β-lactamase. It is likely that 1 inhibits through the same mechanism as O-phenoxy carbonyl-N-(benzyloxy carbonyl]hydroxylamine (L1), while 2, 3, and 4 inhibit through the same mechanism as L2. Thus suggesting that both the polar and nonpolar side groups play a role in the mechanism of the inhibitor.
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Introduction
Structure of the Bacterial Cell Wall

Bacterial cells differ from mammalian cells due to the presence of a highly developed peptidoglycan cell wall located outside the cytoplasmic membrane. The cell wall protects the cell protoplast and provides structural support to the cell that is necessary to prevent against osmotic rupture due to turgor pressure.\textsuperscript{1} Due to its rigidity and high tensile strength, peptidoglycan acts as the scaffold for the bacterial cell.

Peptidoglycan Biosynthesis

Peptidoglycan is composed of repeating alternating N-acetyl glucosamine (NAG) and N-acetylmuramic acid (NAM) subunits that are connected via $\beta$-(1,4)-glycosidic bonds, yielding the glycosidic backbone of the bacterial cell wall.\textsuperscript{2}

As illustrated in Figure 1, peptidoglycan biosynthesis is initiated in the cytoplasm with the MurA-catalyzed synthesis of UDP-N-acetylglucosamine (UDP-NAG) and the MurB-catalyzed synthesis of UDP-N-acetylmuramyl (UDP-NAM) from UDP-GlcNAc. Through a series of ATP-powered ligase reactions, the pentapeptide chain is added to the UDP-NAM to form UDP-NAM-pentapeptide.\textsuperscript{3}

The UDP-NAM-pentapeptide is linked to a transport lipid to form lipid I, and is thus transferred to the cytoplasmic membrane. MurG then catalyzes the addition of UDP-NAG to lipid I to form lipid II. Lipid II is transferred to the outside the cytoplasmic membrane to the cell wall where it is polymerized to form the nascent peptidoglycan strand.\textsuperscript{3}

A nucleophilic serine residue of a DD-transpeptidase attacks the carbonyl carbon atom of the C-terminal D-Ala-D-Ala peptide bond of the NAM-pentapeptide on the nascent
peptidoglycan strand. This attack frees the terminal D-alanine group and yields an acyl-enzyme complex. The nucleophilic amino group from another peptidoglycan strand attacks the acyl-enzyme complex to form a peptide-amide bond, thus yielding the free enzyme and the final cross-linked peptidoglycan strand, which is incorporated into the cell wall. The cross-linking of these peptide chains by DD-transpeptidases gives rise to the three-dimensional structure and high tensile strength of the peptidoglycan layer.

![Diagram of peptidoglycan biosynthesis](image)

**Figure 1:** Biosynthesis of peptidoglycan.
Gram-positive and Gram-negative bacteria

Due to structural differences in the cell wall, bacterial cells are subdivided into Gram-positive and Gram-negative. The cell walls of Gram-positive bacteria (e.g. *Bacillus subtilis, Streptococcus pneumoniae, Staphylococcus aureus*) consist of multiples layers of peptidoglycan with perpendicular lipoteichoic acids, which run through the peptidoglycan layers and anchor to the plasma membrane. The cell walls of Gram-negative bacteria (e.g. *Escherichia coli, Neisseria gonorrhoeae, Salmonella, Enterobacteriaceae*) contain a small layer of peptidoglycan bound to an outer membrane composed of phospholipids and liposaccharides that are often unique to specific bacterial subspecies.1

![Gram-Positive Bacterial Cell Wall](image1.png) ![Gram-Negative Bacterial Cell Wall](image2.png)

**Figure 2:** Comparison between gram-positive and gram-negative cell wall.7
**β-Lactam Antibiotics**

β-Lactam antibiotics are compounds with bactericidal properties characterized by the four-membered β-lactam ring, which act by inhibiting the biosynthesis of the peptidoglycan layer of the bacterial cell wall. These include penicillin derivatives, cephalosporins, monobactams, and carbapenems.⁸

![Penicillin core structure](image)

**Figure 3:** Penicillin core structure.

**Historical Background**

Penicillin was discovered by Alexander Fleming in 1929. Fleming had been culturing colonies of Staphylococcus when one of the plates, untreated by Lysol, had been contaminated by *Penicillium notatum*. Fleming observed that the growth of staphylococci had been inhibited in the area immediately surrounding the mold, suggesting that the mold secreted an antibacterial compound.⁹ The compound was isolated in 1939 through alumina column chromatography by Sir Howard Florey and Ernst Chain. Dorothy Hodgkin determined by X-ray crystallography that the structure of the penicillin molecule contained a four-membered β-lactam ring, fused to a thiazolidine ring.
In 1941, Florey and Chain investigated the clinical use of penicillin to combat bacterial infection in humans. Albert Alexander was the first patient to be treated with penicillin for a Streptococci and Staphylococci infection in his mouth. Following his first dose of penicillin, there was improvement in his condition, although due to an insufficient penicillin supply because of production difficulties, he ultimately died.

*Mechanism of Action of β-Lactam Antibiotics*

β-Lactam antibiotics function by inhibiting peptidoglycan biosynthesis. In 1965, Donald Tipper and Jack Strominger determined that that penicillin is a substrate or transition state analog of the acyl-D-Ala-D-Ala of uncross-linked peptidoglycan.\(^{10}\) The inhibition of the DD-transpeptidase occurs due to the nucleophilic attack on the carbonyl of the β-lactam ring by the serine hydroxyl group in the enzyme active site, thus producing a stable acyl-enzyme.

The covalent bond between the β-lactam antibiotic and the DD-transpeptidase prevents the cross-linking of the stem peptide, thus the peptidoglycan layer of the bacterial cell wall cannot be synthesized. Without the cell wall, the bacterial cell is vulnerable to internal turgor pressure and lysis, eventually leading to cell death.

*Figure 4:* Attack and inactivation of β-lactam antibiotics by β-lactamases.\(^{11}\)
Antibiotic Resistance

Penicillin resistance in bacteria emerged shortly after its clinical introduction in 1941. Due to incomplete treatment of infections and overprescription of antibiotics during World War II, approximately 6% of *S. aureus* produced β-lactamases causing penicillin resistance by 1946. By 1948, over 50% of hospital *S. aureus* were penicillin-resistant and the number has subsequently climbed to about 80–90% worldwide in the 1980s, as shown below in Figure 5. Antibiotic resistance can be transferred between bacteria through conjugation, the transfer of plasmids.

![Graph showing the percentage of antibiotic resistant *S. aureus* since penicillin introduction in 1944](image)

**Figure 5:** Percentage of antibiotic resistant *S. aureus* since penicillin introduction in 1944.
This resistance has given rise to methicillin and vancomycin resistant strains of bacterial infections, which can be lethal. According to the Center for Disease Control (CDC), there were approximately 2,049,442 cases of antibiotic resistant infections in the United States in 2013, of which approximately 23,000 were lethal.13

**β-Lactamases**

β-Lactamases, which are produced by both Gram-negative and Gram-positive bacteria, are hydrolytic enzymes produced by β-lactam resistant bacteria that hydrolyze the amide bond of the β-lactam ring structure. This break yields the antibiotic inert, allowing for the DD-transpeptidase to cross-link the stem peptide of the peptidoglycan.

β-Lactamases are molecularly classified by mechanism of action and conserved amino acid motifs, whereby β-lactamases are subdivided into four groups: A, B, C, and D.14 Classes A, C, and D β-lactamases function via nucleophilic attack on the amide bond of the β-lactam ring by a serine residue in the enzyme active site, as shown below in Figure 6. Class B β-lactamases function using two zinc ions. The first of which coordinates the carbonyl oxygen on the β-lactam ring and thus promotes nucleophilic attack by water, while the other stabilizes negative charge on the nitrogen, thus forming a stable intermediate.15
**Figure 6:** Hydrolysis of a β-lactam by β-lactamase

**β-lactamase Inhibitors**

Knowles proposed two strategies to combat β-lactamases.\(^6\) The first strategy relies on the design of novel β-lactam antibiotics that are resistant to hydrolysis by β-lactamases by altering their structure while maintaining antibiotic properties. This strategy became viable with the discovery and subsequent synthesis of 6-aminopenicillanic acid and 7-aminocephalosporanic acid. While these compounds showed significant resistance to β-lactamases, they were not as effective as β-lactam antibiotics against bacteria.\(^6\) The second strategy involves the neutralizing the β-lactamase with a β-lactamase inhibitor, and treating the bacterial infection using conventional β-lactam antibiotics.

Most β-lactamase inhibitors belong to two major groups: transition-state analog inhibitors and mechanism-based inhibitors. Transition-state analog inhibitors function by binding to the enzyme through specific interaction at the active site that would be expected from the natural substrate in the transition state. Examples of transition-state analogs include boronates and
phosphonates, which are effective because of their ability to generate tetrahedral geometry and an anionic moiety at the active site.\textsuperscript{17}

Mechanism-based inhibitors have structural similarities to the native substrates and react similarly with enzyme; however, the inhibitor also becomes chemically modified by the enzyme in such a way to inactivate it. These inhibitors most often form covalent bonds to the enzyme active site, which neutralize the enzyme. However, traditional mechanism-based inhibitors are often \( \beta \)-lactams and are therefore also susceptible to \( \beta \)-lactamase catalyzed hydrolysis.\textsuperscript{18}

Therefore, it is useful to investigate non-\( \beta \)-lactam alternatives as \( \beta \)-lactamase inhibitors.

\textbf{Scheme 1:} Reaction pathway for a mechanism-based inhibitor.

The reaction pathway for mechanism-based inhibitors is depicted above in Scheme 1 where \( E \) represents the free enzyme and \( I \) represents the inhibitor. \( EI_i \) is the non-covalent Michaelis complex, \( E_i \) is the acyl-enzyme intermediate, and \( EI_i' \) is the modified acyl-enzyme complex. \( E_i \) can undergo normal turnover, as represented by \( k_3 \), resulting in the free enzyme and product. \( E_i \) may also be converted into the modified acyl-enzyme complex, \( EI_i' \), by pathway \( k_4 \) which leads to partial or complete inhibition of the enzyme.
O-Aryloxy-carbonyl Hydroxamates

Research conducted by Pauline Wyrembak in the Pratt Laboratory developed the first generation O-aryloxy-carbonyl hydroxamate inhibitors from knowledge of the mechanism of the reaction of β-lactamases with a depsipeptide substrate, phosphonate, and vanadate inhibitors such as those shown below in Figure 7.\textsuperscript{19}

![Figure 7: Phosphonate inhibitor (right)\textsuperscript{17} and vanadate inhibitor (left).\textsuperscript{20}](image)

Compound \textbf{L1} derived from these, contains two potential leaving groups and irreversibly covalently inhibits class A and class C β-lactamases by cross-linking Ser 64 with Lys 315.\textsuperscript{19} The reaction mechanism is shown below in Scheme 2.

![Scheme 2: Mechanism of action of \textbf{L1} with enzyme to form cross-linking at active site (red) or turnover (green).\textsuperscript{19}](image)
A second generation inhibitor, compound L2 was discovered by Dr. Ronak Tilvawala. Compound L2 was an effective inhibitor against the class C P99 β-lactamase by forming a hydroxamate acyl-enzyme complex, stabilized by the Tyr 150, Ser 212, and Arg 204 residues in the Ω-loop of the active site as shown below in Scheme 3.²¹

![Scheme 3: Mechanism of action of L2 with enzyme to form hydroxamate acyl enzyme complex.](image)
Research Aims

Based on previous work done by members of the Pratt lab, the goal of this thesis was to design and synthesize other analogues of the O-aryloxycarbonyl hydroxamates in order to elucidate the importance of the polar and nonpolar groups on the hydroxamic acid side chain of the inhibitor. We therefore prepared and investigated the β-lactamase inhibitory properties of the following O-aryloxycarbonyl hydroxamates. Their capabilities as inhibitors of Enterobacter cloacae P99 β-lactamase were evaluated through kinetic studies. We therefore synthesized and investigated the inhibitory properties of the following O-aryloxycarbonyl hydroxamates.
Materials and Methods
Materials

Syntheses

Solvents including ethyl acetate, toluene, and dichloromethane (DCM) were purchased from Pharmco. Hexanes and sodium sulfate were purchased from Macro Fine Chemicals. Hydrochloric acid was purchased from Pharmco. Benzene was purchased from EMD chemicals. Triethylamine was purchased from Fluka Chemica. Phenyl chloroformate and imidazole were purchased from Sigma Aldrich. Pyridine was purchased from Spectrum. Thin layer chromatography plates were purchased from AnalTech. 1,1’-carbonyldiimidazole (CDI), butyl chloroformate, hydroxylamine hydrochloride, and delta-valerolactone were purchased from Acros Organics. 1-Amino-4-butanol was purchased from Chem-Impex International Incorporated. O-benzylhydroxylamine hydrochloride was purchased from Aesar. Magnesium sulfate was purchased from Macron Chemicals.

The 1H NMR spectra were obtained using a Varian 300 MHz spectrophotometer and the IR spectra were obtained using a Perkin Elmer Spectrum BX FT-IR System. The high resolution mass spectra of 1, 2, 3, and 4 were obtained from the Mass Spectrometry Laboratory, University of Illinois at Urbana-Champaign.

Kinetics

The class C P99 β-lactamase from Enterobacter cloacae was purchased from the Centre for Applied Microbiology and Research in Porton Down, Wiltshire, United Kingdom. Bovine serum albumin (BSA) and MOPS buffer were purchased from Sigma Aldrich. Absorption
spectra and spectrophotometric reaction rates were measured with a Hewlett Packard 8453 UV spectrophotometer.
**Syntheses**

**Synthesis of 1**

![Chemical structure of A1 and A2](image)

**Scheme 4: Synthesis of 1**

Intermediate A1: A solution of O-benzylhydroxylamine hydrochloride (1.48 g, 12.08 mmol) and pyridine (2.22 g, 28.16 mmol) in dichloromethane (60 mL) was added dropwise under a nitrogen atmosphere at 0°C to a solution of 4-butyl chloroformate (1.65 g, 12.08 mmol) in DCM (120 mL). The reaction mixture was stirred for three hours and slowly brought back to room temperature. The organic layer was evaporated to dryness and the residues were dissolved in 50:50 ethyl acetate:ether. The organic layer was washed with 1 M HCl, dried over magnesium sulfate, and evaporated to dryness to obtain A1 as a colorless oil. Yield: (1.41 g, 6.33 mmol, 52% yield) The obtained product was used for the next step without further purification.

$^1$H NMR (CDCl3, 300 MHz): 0.95 (t, 3H, J = 3.0 Hz), 1.41 (quint, 2H), 1.66 (hext, 2H), 4.14 (m, 2H), 4.85 (s, 2H), 7.18 (s, 1H, -NH), 7.38 (s, 5H).
Intermediate A2: Compound A1 (1.41 g, 6.33 mmol) and Pd/C (0.2 g, 0.633 mmol) catalyst were added to ethanol (20 mL). The reaction was hydrogenated at 45 psi for 4 hours. The catalyst was filtered off and the filtrate was concentrated using rotary evaporation to obtain A2 as a colorless oil. Yield: (0.546 g, 4.1 mmol, 64% yield).

1H NMR (DMSO, 300 MHz): 0.88 (t, 3H, J = 6.2 Hz), 1.32 (m, 2H), 1.54 (m, 2H), 4.00 (m, 2H), 8.65 (s, 1H, -NH), 9.54 (s, 1H, -OH).

Compound 1: Imidazole (0.206 g, 4.1 mmol) in DCM (3.3 mL) was added to A2 (0.546 g, 4.1 mmol) in DCM (8 mL) under a nitrogen atmosphere at 0°C. The mixture was stirred for 20 minutes. A solution of phenyl chloroformate (0.46 g, 4.1 mmol) in DCM (1.6 mL) was added dropwise to the reaction mixture over one minute. The mixture was stirred for 45 minutes under nitrogen atmosphere at 0°C. The mixture was filtered. To the filtrate, ethyl acetate (10 mL) was added to form a white precipitate. The precipitate was filtered and the filtrate was evaporated to dryness and dried in the oil pump overnight. The crude product was purified by chromatography on silica gel (90:10 hexane:ethyl acetate), yielding 1 as a yellow oil (0.164 g, 14% yield).

1H NMR (DMSO, 300 MHz): 1.18 (t, 3H, J = 6.18 Hz), 1.33 (m, 2H), 1.69 (m, 2H), 4.00 (m, 2H), 7.40 (m, 5H), 11.56 (s, 1H, -NH)

TOF MS m/z [M]+: 276.0840; C_{12}H_{15}NO_{5} requires 276.0848

IR (cm⁻¹) ATR: 759.45 (aromatic C-H bend), 1224.83 (C-O stretch), 1494.15 (alkane C-H bend), 1592.68 (aromatic C=C stretch), 1731.84 (C=O stretch), 1798.95 (C=O stretch), 2875.64 (aromatic C-H stretch) 2963.89 (alkane C-H stretch), 3269.68 (amide -NH stretch).
Synthesis of 2

Intermediate B1: 2-Phenylethanol (2.06 g, 16.83 mmol) in DCM (62 mL) was added to a pre-cooled triphosgene solution (5.00 g, 16.83 mmol) in DCM (31 mL) under nitrogen atmosphere at -20°C and stirred for 10 minutes. Triethylamine (2.04 g, 20.21 mmol) in DCM (9.3 mL) was added to the reaction mixture and stirred to room temperature for 4 hours. The reaction was evaporated to dryness and the resulting residues were evaporated in ethyl acetate (60 mL). The reaction was filtered, evaporated to dryness, and dried overnight on the oil pump. The obtained product was used for the next step without further purification.

\[ ^1H \text{NMR (CDCl}_3, 300 \text{MHz): 3.05 (t, 2H, } J = 6.9 \text{ Hz), 4.51 (t, 2H, } J = 7.2 \text{ Hz), 7.30 (m, 5H).} \]
IR (cm⁻¹) ATR: 699.33 (aromatic C-H bend), 1146.65 (C-O stretch), 1455.44 (aromatic C=C stretch), 1777.38 (acid chloride C=O stretch), 3030.94 (aromatic C-H stretch).

Intermediate **B2**: O-Benzylhydroxylamine hydrochloride (2.8 g, 16.83 mmol) and pyridine (3.06 g, 39.14 mmol) in DCM (83 mL) was added dropwise under nitrogen atmosphere at 0°C to **B1** (3.1 g, 16.83 mmol) in DCM (167 mL),. The reaction mixture was stirred for three hours and slowly brought back to room temperature. The organic layer was evaporated to dryness and the resulting residues were dissolved in 50:50 ethyl acetate:ether. The organic layer was washed with 1 M HCl, dried over magnesium sulfate, and evaporated to dryness to obtain the crude product (3.58 g, 13.20 mmol, 78% yield). The obtained product was purified by chromatography on silica gel (90:10 hexane:ethyl acetate), yielding the product **B2** as a yellow oil. Yield: (1.00 g, 3.69 mmol, 22.34% yield).

**¹H NMR (DMSO, 300 MHz)**: 2.89 (t, 2H, J = 6.8 Hz), 4.22 (t, 2H, J = 5.1 Hz), 4.68 (s, 2H), 7.29 (m, 10H), 10.34 (s, 1H).

Intermediate **B3**: Compound **B2** (1.00 g, 3.69 mmol) was dissolved in ethanol (20 mL). Pd/C (0.49 g, 0.369 mmol) catalyst was added and the reaction was hydrogenated at 45 psi for 4 hours. The catalyst was filtered off, the filtrate was concentrated using rotary evaporation, and the residue was dried overnight on the oil pump. The residue was then washed with ethyl acetate and the precipitate was dried to obtain **B3** as a white solid. Yield: (0.50 g, 2.76 mmol, 74.8% yield).

**¹H NMR (DMSO, 300 MHz)**: 2.89 (t, 2H, J = 6.6 Hz), 4.16 (t, 2H, J = 7.2 Hz), 7.24 (m, 5H), 8.64 (s, 1H, -NH), 9.62 (s, 1H, -OH)
Product 2: Imidazole (0.11 g, 2.2 mmol) in DCM (3 mL) was added to B3 (0.40 g, 2.2 mmol) in DCM (7 mL) under nitrogen atmosphere at 0°C and was stirred for 20 minutes. A solution of phenyl chloroformate (0.25 g, 2.2 mmol) in DCM (1.5 mL) was added dropwise over one minute to the reaction mixture. The mixture was stirred for 45 minutes under nitrogen atmosphere at 0°C. The mixture was filtered. Ethyl acetate (10 mL) was added to the filtrate to form a white precipitate. The precipitate was filtered off and the filtrate was evaporated to dryness and dried in the oil pump for 4 hours. The crude product was purified by recrystallization (98:2 cyclohexane:benzene), yielding the product B5 as a white solid (76 mg, 11.3% yield).

$^1$H NMR (DMSO, 300 MHz): 2.89 (t, 2H, $J = 6.7$ Hz), 4.31 (t, 2H, $J = 7.1$ Hz), 7.26 (m, 10H), 11.58 (s, 1H).

TOF MS $m/z$ [M]$^+$: 324.0842; $C_{16}H_{15}NO_3Na$ requires 324.0848

IR (cm$^{-1}$) ATR: 751.65 (aromatic C-H bend), 1221.50 (C-O stretch), 1494.72 (aromatic C=C stretch), 1721.88 (C=O stretch), 1839.71 (C=O stretch), 3224.25 (N-H stretch).
Synthesis of 3

Intermediate **C1**: delta-Valerolactone (18.60 g, 189 mmol) and potassium hydroxide (10.60 g, 189 mmol) were dissolved in water (30 mL). The reaction mixture was refluxed for three hours at 70°C. The resulting salt was dissolved in excess water, filtered, and evaporated to dryness. The salt was suspended in toluene (40 mL) and evaporated to dryness. The crude product was recrystallized (95% ethanol) and dried on the oil pump overnight to yield C1. Yield: (27.63 g, 177 mmol, 94% yield).

$^1$H NMR (D2O, 300 MHz): 1.408 (br, 4H), 2.065 (t, 2H, J = 7.2 Hz), 3.449 (t, 2H, J = 5.7 Hz).
Intermediate **C2**: **C1** (18.05 g, 0.116 mol), 4-methoxybenzyl chloride (15.77 g, 0.116 mol), and sodium iodide (332.67 mg, 2.22 mmol) were dissolved in dimethylformamide (400 mL) and stirred at room temperature for 24 hours. The reaction mixture was evaporated to dryness and dried on the oil pump overnight. The resulting residue was dissolved in dichloromethane (125 mL) and washed with a saturated solution of sodium bicarbonate and water. The organic layer was separated and dried over magnesium sulfate. The product was then filtered and the filtrate was concentrated using rotary evaporation to obtain **C2** as a yellow oil. Yield: (27.64 g, 23% yield).

\[^1\text{H }\text{NMR (DMSO, 300 MHz): 1.41 (m, 2H), 1.56 (m, 2H), 2.31 (t, 2H, J = 4.9 Hz), 3.37 (m, 2H), 3.77 (s, 3H), 4.41 (t, 1H, -OH, J = 3.6 Hz), 5.04 (s, 2H), 7.00 (d, 2H, J = 7.2 Hz), 7.36 (d, 2H, J = 9.1 Hz).}\]

Intermediate **C3**: To a solution of **C2** (1.00 g, 4.2 mmol) in acetonitrile (20 mL), CDI (1.05 g, 6.3 mmol) was added and stirred at room temperature for 90 minutes. To the reaction mixture, imidazole (1.14 g, 16.8 mmol) and hydroxylamine hydrochloride (1.45 g, 21.0 mmol) were added and stirred for 4 hours to completion. The imidazole hydrochloride precipitate was filtered out of the reaction mixture and the filtrate was dissolved in ethyl acetate and 0.2 M citric acid. The organic layer was washed with a brine solution, dried over sodium sulfate, and evaporated to dryness to obtain **C3** as a colorless oil. Yield: (0.74 g, 2.5 mmol, 60% yield) The obtained product was used for the next step without further purification.
$^1$H NMR (DMSO, 300 MHz): 1.54 (m, 4H), 2.31 (t, 2H, J = 4.8 Hz), 3.73 (s, 3H), 3.95 (t, 2H, J = 3.6 Hz), 4.38 (q, 2H, J = 5.1 Hz), 4.98 (s, 2H), 6.92 (d, 2H, J = 6.3 Hz), 7.28 (d, 2H, J = 9.0 Hz), 8.62 (s, 1H, -NH), 9.51 (s, 1H, -OH).

Intermediate C4: Imidazole (0.13 g, 2.50 mmol) was added to C3 (0.74 g, 2.5 mmol) in dichloromethane (10 mL) under nitrogen atmosphere at 0°C. The mixture was stirred for 20 minutes. To the reaction mixture, a solution of phenyl chloroformate (0.31 g, 2.5 mmol) in dichloromethane (1.5 mL) was added dropwise over one minute. The mixture was stirred for 45 minutes under nitrogen atmosphere at 0°C. The mixture was filtered. To the filtrate, ethyl acetate (10 mL) was added to form a white precipitate. The precipitate was filtered and the filtrate was evaporated to dryness and dried in the oil pump overnight, yielding the crude product as a yellow oil (1.04 g). The crude product was purified by chromatography on silica gel (90:10 hexane:ethyl acetate), yielding the product C4 as a colorless oil (57.6 mg, 59% yield).

$^1$H NMR (DMSO, 300 MHz): 1.58 (m, 4H), 2.34 (t, 2H, J = 6.7 Hz), 3.74 (s, 3H), 4.11 (t, 2H, J = 5.3 Hz), 4.99 (s, 2H), 7.35 (m, 9H), 11.54 (s, 1H)

Product 3: TFA (1 mL) was added dropwise under nitrogen at room temperature to the solution of 100 mg of C4 in DCM (1 mL). The reaction was monitored by TLC to completion. Excess solvent was removed via rotary evaporation, yielding the product 3 as a colorless solid (72 mg, 100% yield).

$^1$H NMR (DMSO, 300 MHz): 1.57 (m, 4H), 2.23 (t, 2H, J = 6.9 Hz), 4.12 (t, 2H, J = 5.3 Hz), 7.35 (m, 5H), 11.55 (s, 1H).
TOF MS $m/z$ [M]$^+$: 320.0739; C$_{13}$H$_{17}$NO$_2$Na requires 320.0746

IR (cm$^{-1}$) ATR: 777.72 (aromatic C-H bend), 1043.20 (C-O stretch), 1215.37 (C-O stretch), 1495.96 (aromatic C=C stretch), 1757.01 (C=O stretch), 1794.31 (C=O stretch), 2354.15, 2975.92 (alkane C-H stretch), 3283.53 (N-H stretch).
Synthesis of 4

Intermediate D1: To a pre-cooled solution of 1-amino-4-butanol (5.00 g, 56.1 mmol) and triethylamine (8.50 g, 84 mmol) in 66% aqueous dioxane (225 mL), a solution of di-tert-butyl dicarbonate (13.99 mL, 61.6 mmol) in dioxane (75 mL) was added and the mixture was stirred at room temperature for 4 hours. The solvent was removed by rotary evaporation and the resulting residue was partitioned between ethyl acetate and 10% citric acid. The product was extracted from the aqueous layer with ethyl acetate three times and the combined organic layer was washed with a 5% sodium bicarbonate solution and dried over magnesium sulfate. The crude product (10.81 g) was purified by fractional distillation using the Kugelrohr apparatus at 130 °C, yielding D1 as a yellow oil (8.20 g, 43.3 mmol, 77% yield).

\(^1\)H NMR (DMSO, 300 MHz): 1.35 (s, 9H), 2.87(q, 4H), 3.35 (q, 4H), 4.34 (t, 1H, -OH, J = 5.3 Hz), 6.75 (t, 1H, J = 3.4 Hz).
Intermediate **D2**: To **D1** (1.00 g, 5.3 mmol) in acetonitrile (20 mL), CDI (1.28 g, 7.95 mmol) was added and stirred at room temperature for 90 minutes. To the reaction mixture, imidazole (1.44 g, 21.2 mmol) and hydroxylamine hydrochloride (1.82 g, 26.5 mmol) were added and stirred for 4 hours to completion. The imidazole hydrochloride precipitate was filtered out of the reaction mixture and the filtrate was partitioned between ethyl acetate and 0.2 M citric acid. The organic layer was washed with a brine solution, dried over sodium sulfate, and evaporated to dryness to obtain **D2** as a colorless oil. Yield: (1.10 g, 4.4 mmol, 83% yield) The obtained product was used for the next step without further purification.

$^1$H NMR (DMSO, 300 MHz): 1.35 (s, 9H), 1.37 (m, 2H) 1.46 (q, 2H, J = 8.1 Hz), 2.90 (q, 2H, J = 5.9 Hz), 3.96 (t, 2H, J = 2.9 Hz), 6.76 (t, 1H, -NH), 8.62 (s, 1H, -NH), 9.48 (s, 1H, -OH).

Intermediate **D3**: Imidazole (0.299 g, 4.4 mmol) was added to **D2** (1.10 g, 4.4 mmol) in dichloromethane (10 mL) under a nitrogen atmosphere at 0°C. The mixture was stirred for 20 minutes. To the reaction mixture, a solution of phenyl chloroformate (0.495 g, 4.4 mmol) in dichloromethane (2 mL) was added dropwise over one minute. The mixture was stirred for 2 hours under nitrogen atmosphere at 0°C. The mixture was filtered. To the filtrate, ethyl acetate (10 mL) was added to form a white precipitate. The precipitate was filtered and the filtrate was evaporated to dryness and dried in the oil pump overnight, yielding the crude product as a colorless solid (1.34 g). The crude product was purified by chromatography on silica gel (80:20 hexane:ethyl acetate), yielding **D3** as a yellow oil (426.5 mg, 1.12 mmol, 25% yield).
$^1$H NMR (DMSO, 300 MHz): 1.35 (s, 9H), 1.40 (m, 4H), 1.58 (q, 2H, J = 5.4 Hz), 2.91 (q, 2H, J = 5.8 Hz), 4.15 (t, 2H, J = 5.3 Hz), 6.85 (t, 1H, -NH), 7.26 (d, 2H), 7.32 (t, 1H), 7.47 (t, 2H), 11.53 (s, 1H, -NH).

IR (cm$^{-1}$) ATR: 688.44, 719.77, 777.65, 1116.82, 1213.34, 1367.13, 1493.94, 1518.91, 1689.14, 1757.08, 1804.58 (C=O stretch), 2975.18 (C-H stretch), 3210.70 (-NH stretch), 3359.50 (-NH stretch)

Product 4: TFA (1 mL) was added dropwise under nitrogen at room temperature to the solution of 100 mg of D4 in dichloromethane (1 mL). The reaction was monitored by TLC to completion at 1 hour. Excess solvent was removed via rotary evaporation, yielding the trifluoroacetate salt of product D5 as a yellow oil (67 mg, 100% yield).

$^1$H NMR (DMSO, 300 MHz): 1.64 (m, 4H), 2.49 (q, 2H, J = 1.5 Hz), 4.18 (t, 2H, J = 6.3 Hz), 7.26 (d, 2H, J = 6.2 Hz), 7.32 (t, 1H, J = 8.1 Hz), 7.47 (t, 2H, J = 7.8 Hz), 7.66 (br s, 3H, NH$_3^+$), 11.58 (s, 1H, -NH).

TOF MS m/z [M$^+$]: 269.1136; C$_{12}$H$_{17}$N$_2$O$_3$ requires 269.1137

IR (cm$^{-1}$) ATR: 723.45 (aromatic C-H bend), 780.43 (aromatic C-H bend), 1207.28 (C-O stretch), 1492.23 (aromatic C=C stretch), 1678.71 (amide C=O stretch), 1743.65, 1799.51 (C=O stretch), 2966.87 (-CH stretch), 3163.29 (-NH stretch).
Analytical and Kinetic Methods

The concentration of P99 β-lactamase stock solution was measured spectrophotometrically, assuming its extinction coefficient to be 7.1 x 10^4 M^-1 s^-1 at 280 nm. All kinetics experiments were performed at 25 °C in 20 mM MOPS buffer, pH = 7.5.

Reactions in aqueous solution

The rate of spontaneous hydrolysis reaction of 1, 3, and 4 were measured spectrophotometrically at λ = 270 nm at 25 °C in for 3600 seconds. 5 microliters of 10 mM 1, 3, and 4 in DMF was added to 995 microliters of 20 mM MOPS buffer, pH = 7.5. The first order rate constant for the hydrolysis of compounds 1, 3, and 4 was measured by fitting the spectroscopic data in Kaleidagraph using the following equation:

\[ Y = m1 + (m2-m1) \times \exp(-m3*m0) \]

Where \( y \) = absorbance at time \( t \), \( m0 \) = time, \( m1 \) = initial absorbance, \( m2 \) = final absorbance, and \( m3 \) = rate constant.

Time-Based Inactivation Assay

The time-based inactivation of β-lactamase by compounds 1, 2, 3, and 4 was measured at 25 °C. P99 enzyme (150 nM) was mixed and incubated with 1000 nM inhibitor and 500 nM inhibitor for 1, 2, 3, and 4. The reaction mixture (20 μL aliquots) was then added to cuvettes containing 980 μL of 20 mM MOPS buffer and 300 μM cephalothin substrate. The initial rates of hydrolysis of the substrate cephalothin (monitored at 270 nm) were measured at various points in
time until the enzyme appeared to have been as inhibited as possible. The initial rates were plotted against time and fit using Scheme in Dynafit to determine the kinetic parameters.

\[
\begin{align*}
E + I & \rightarrow EI \\
I & \rightarrow P
\end{align*}
\]

Scheme 8

**Concentration-Based Inactivation Assay**

The concentration-based inactivation of compounds 1, 2, 3, and 4 was measured at 25 °C. P99 enzyme (150 nM) was incubated in MOPS buffer containing 1 mg/mL BSA with 0 μM, 0.01 μM, 0.02 μM, 0.05 μM, 0.10 μM, 0.20 μM, 0.5 μM, and 1.00 μM inhibitor. The reaction mixture was incubated overnight. The reaction mixtures (20 μL aliquots) were added to cuvettes containing 980 μL of 20 mM MOPS buffer and 300 μM cephalothin substrate. The initial rates were plotted against time.
Results and Discussion
Synthesis of I

Scheme 9: Acylation of 4-butyl chloroformate to form A1

The method used to synthesize A1 was based on the procedure outlined by Tilvawala and Pratt, as shown above in Scheme 9. A $^1$H NMR of the starting material, O-benzylhydroxylamine hydrochloride in CDCl$_3$ and DMSO contains sharp singlet peaks at 5.17 ppm for the two methylene hydrogens and 7.42 ppm for the five aromatic hydrogens, as well as a broad singlet peak at 11 ppm for the ammonium hydrogens. The successfully acylated product, A1, would not contain a peak at 11 ppm due to the upfield shift caused by the loss of positive charge of the NH$_3^+$. The peak from the newly formed amide was predicted between 5.5 - 8.0 ppm.

$^1$H NMR was used to determine if A1 was successfully synthesized. The spectrum was taken in CDCl$_3$ and indicated that there was no residual starting material due to the absence of the peaks for the starting hydroxamic acid at 5.17 ppm, 7.42 ppm, and 11 ppm. Moreover, a singlet peak at 7.18 ppm with an integration of one proton met the requirements for the acylated NH of the product. The additional peaks were similarly assigned. A triplet peak at 0.95 ppm with an integration of three protons corresponded to the methyl group found at the end of the butyl side chain. Peaks at 1.41 ppm, 1.66 ppm, 4.14 ppm, each with integrations of two hydrogens were assigned to the methylene protons of the butyl side chain. The increasing downfield shifts
of the protons in the $^1$H NMR from the butyl side chain is due to the deshielding caused by the increasing proximity to the oxygen. The singlet peak at 4.85 ppm was determined to belong to the benzylic hydrogens. The presence of the anticipated aromatic group was confirmed at 7.38 ppm.

Minor impurities were detected at 1.23 ppm and 2.19 ppm. These peaks were thought to belong to residual butyl chloroformate that had not been extracted in the hydrochloric acid wash or a dibutyl carbonate that formed as a byproduct. However, due to the relative purity of this product, the synthesis proceeded without additional purification at this step.

![Scheme 10: Hydrogenation of A1 to synthesize A2](image)

**Scheme 10: Hydrogenation of A1 to synthesize A2**

Compound A1 was then hydrogenated at 45 psi for 4 hours using Pd/C catalyst in ethanol as shown in Scheme 10. To corroborate the synthesis of A2, $^1$H NMR was used to determine the structure of the product. The results from the $^1$H NMR indicated the successful synthesis of A2. Unlike A1, the fully hydrogenated A2 no longer contained the aromatic hydrogen peaks at 7.38 ppm, nor did it contain the singlet peak at 4.85 ppm associated with the benzylic hydrogens. Moreover, the spectrum of A2 included a downfield shifted singlet for the amide hydrogen at 8.65 ppm, as well as a singlet peak for the hydroxyl hydrogen at 9.54 ppm.
The final step of the synthesis to make 1 utilized the method of Pelto and Pratt for O-aryloxycarbonyl hydroxamates.\textsuperscript{24} A2 was reacted with imidazole and phenyl chloroformate in DCM under nitrogen atmosphere at 0°C for 45 minutes. The imidazole acts as a base to neutralize the hydrochloric acid byproduct and form an imidazole hydrochloride precipitate.

The \textsuperscript{1}H NMR of the crude product indicated the successful synthesis of 1. However, there were impurities from phenol at 6.74 ppm, 7.13 ppm, and 7.67 ppm (-OH singlet), as well as additional butyl groups indicated by triplets at 3.98 ppm and 4.25 ppm. Unidentified impurities were also indicated at 9.1 ppm and 9.45 ppm. The crude product was therefore purified by flash column chromatography on silica gel from 90:10 hexane:ethyl acetate, yielding the product 1 as a yellow oil.

To corroborate the successful synthesis of 1, a \textsuperscript{1}H NMR spectrum of the resulting product in DMSO (Figure A) indicated the formation of the acylated product due disappearance of the hydroxyl peak at 9.54 ppm in the \textsuperscript{1}H NMR of A2. Moreover, the presence of peaks in the aromatic region that integrate for five protons indicates the successful addition of the phenyl group. Moreover, the amide hydrogen found at 8.65 ppm in A2 has shifted downfield to 11.49 ppm as a result of significant deshielding caused by the addition electronegative oxygen atoms. This shift is typical of those seen in the formation of O-acyl hydroxamates.\textsuperscript{24} The \textsuperscript{1}H NMR contained the peaks indicative of the butyl side chain at 0.95 ppm, 1.3 ppm, 1.6 ppm, and 4.15 ppm. Minor impurities were detected in a 1:10 ratio with the product.

To further support the formation of 1, an IR spectrum was taken. The results shown in Figure 9 included a peak at 1789 cm\textsuperscript{-1}, which corresponds to the literature values for the characteristic carbonyl of O-acyl hydroxamates at 1800 cm\textsuperscript{-1}. Moreover, a TOF ESI\textsuperscript{+} mass
spectrum was taken to verify these results, as shown in Figure 10. The mass spectrum revealed a peak at 276.0840 amu. The chemical formula for 1 is C_{12}H_{15}NO_{5}, which gives a calculated molecular weight of 276.0848 amu. Several higher molecular mass peaks are also evident in the mass spectrum. These may arise from the impurity peaks seen in the NMR spectrum. Additionally, the mass spectrometer operator seemed to have difficulty with these O-acyl hydroxamates, perhaps because of their labile nature, and on one occasion at least had to add NaCl to find a relevant peak. Based on the ^1H NMR, IR, and mass spectral data, it was concluded that 1 was successfully synthesized.

Figure 8: ^1H NMR spectrum of 1 in DMSO
Figure 9: ATR IR Spectrum of 1
Figure 10: Mass Spectrum of 1
Synthesis of 2

Compound 2 strongly resembles the lead compound designed by Pauline Wyrembak in 2003. This structural analog differs from L1 due to the presence of an additional methylene group before the terminal aryl group on the hydroxamic acid side chain. This compound also serves as a structural analog of compound 1.

![Scheme 1](image)

**Scheme 1:** Intended synthesis of B1.

Initial attempts at synthesizing B1 shown above in Scheme 1, were based on the procedure outlined by Ronak Tilvawala. The commercially available 2-phenylethanol and DMAP in a 1:1 ratio was added dropwise at 0°C to a stirred solution of phosgene in DCM and was reacted for 2 hours under nitrogen atmosphere. Upon analysis of the product using IR spectroscopy, it was determined that the synthesis of the product was unsuccessful due to the lack of an acid chloride C=O stretch.

In a second attempt to synthesize B1, 2-phenylethanol was added to a pre-cooled triphosgene solution in DCM under nitrogen atmosphere at -20°C in an ethanol and ethylene glycol bath with dry ice for 10 minutes. Triethylamine was then added and the reaction mixture was stirred for 4 hours. The reaction was evaporated to dryness and the resulting residues were
extracted with ethyl acetate. The ethyl acetate solution was filtered and the ethyl acetate was removed by evaporation, yielding B1.

The synthesis of B1 was confirmed with 1H NMR in CDCl3. The initial spectrum of 2-phenylethanol contains similar peaks to that of B1, however the spectrum B1 is shifted downfield due to the deshielding caused by the addition of the strongly electronegative oxygen and chlorine. The 1H NMR was consistent with the successful synthesis of B1, but, due to the similarity in structure between the starting material and the product, the spectrum alone was inconclusive. The spectrum also contained residual triethylammonium hydrochloride based on peak a triplet peak at 1.27 ppm, a triplet at 3.05 ppm, and a quartet peak at 4.13 ppm.

To corroborate the synthesis of B1, an ATR IR spectrum of the product was taken. The spectrum contained the characteristic acid chloride C=O stretch at 1777.38 cm⁻¹ with a corresponding C-O stretch at 1146.65 cm⁻¹, indicating the successful formation of B1. Due to the high reactivity of B1, the synthesis proceeded without additional purification at this step.

As shown below in Scheme 11, B1 was reacted with O-benzylhydroxylamine hydrochloride and pyridine under nitrogen atmosphere at 0°C for three hours as described in the methods section.

Scheme 11: Formation of B2 from B1
\(^1\)H NMR of the crude product indicated the formation of the product due to the benzylic hydrogen peak at 4.68 ppm as well as the amide hydrogen peak at 10.34 ppm. This was also corroborated by the triplet peaks at 2.89 ppm and 4.22 ppm, which corresponded to the anticipated methylene protons in \textbf{B2}. However, triplet peaks at 3.0 ppm and 3.82 ppm indicated additional methylene protons, beyond those expected for \textbf{B2}. Moreover, the integration for the aromatic region, expected to be ten based on the structure of \textbf{B2}, had an integration of twenty. For this reason, it was predicted that the impurity in the \(^1\)H NMR spectrum was likely the structure shown below in Figure 11. Phenol was also a possible byproduct.

![Chemical Structure](image)

**Figure 11**: Predicted impurity of crude reaction mixture for \textbf{B2}.

Thin layer chromatography in 90:10 hexane:ethyl acetate yielded 3 spots with retention factors of 0.11, 0.33, and 0.44. The crude reaction mixture was then dissolved in 90:10 hexane:ethyl acetate and purified by flash column chromatography on silica gel, yielding the product \textbf{B2} as a yellow oil.

\(^1\)H NMR confirmed the purification of \textbf{B2} in the collected fractions based on the disappearance of the triplet peaks at 3.0 ppm and 3.82 ppm. The presence of the methylene triplets at 2.89 ppm and 4.22 ppm both with an integration of two protons indicated that the fraction did not contain any additional products with methylene protons. The removal of phenol
from the compound was indicated by the integration for ten protons under the multiplet at 7.29 ppm. Moreover, the singlet peaks at 4.68 ppm and 10.34 ppm corresponded to the diagnostic benzylic hydrogen peak and the amide hydrogen peak respectively.

**B2** was then hydrogenated at 45 psi for 4 hours using Pd/C as a catalyst. Much as for the synthesis of **A2**, **¹H NMR** was used to confirm the synthesis of **B3**. The fully hydrogenated **B3** no longer contained the aromatic hydrogen peaks at 7.29 ppm, nor did it contain the singlet peak at 4.68 ppm associated with the benzylic hydrogens. Moreover, the spectrum of **B3** included an downfield shifted singlet for the amide hydrogen (integration of one proton) at 8.64 ppm, as well as a singlet peak for the hydroxyl hydrogen (integration of one proton) at 9.62 ppm. The **¹H NMR** indicated that **B3** was pure and therefore did not require additional purification at this step.

**B3** was reacted with phenyl chloroformate and imidazole in DCM under nitrogen atmosphere at 0°C for 45 minutes under nitrogen atmosphere at 0°C. The crude product was purified by recrystallization from 98% cyclohexane and 2% benzene, yielding **2** as a white solid.

**¹H NMR** was used to determine if **2** was successfully synthesized. Unlike the spectrum of **B3**, the spectrum of **2** (Figure 12) included trace amounts (1:5 ratio) the amide hydrogen singlet at 8.64 ppm, and the singlet peak for the hydroxyl hydrogen at 9.62 ppm, suggesting that **B3** had been mostly reacted with the phenyl chloroformate and imidazole. The diagnostic peak at 11.58 ppm indicated that the hydroxamid -NHOH had been successfully O-acylated by the phenyl chloroformate, causing the downfield shift of the NH in the **¹H NMR**. Moreover, the spectrum indicated a multiplet in the aromatic region, 7.26 ppm, with an integration of ten protons to indicate both phenyl rings in the final product. The spectrum also contained the anticipated
methylenes peaks at 2.89 ppm and 4.31 ppm. As predicted, both peaks integrated for two protons. Analysis of the $^1$H NMR therefore indicated the successful synthesis of compound 2.

To support the formation of 2, an IR spectrum was taken as shown in Figure 13 and had the anticipated peak at 1839.71 for the O-acyl hydroxamate carbonyl. A TOF ESI$^+$ mass spectrum, shown in Figure 14, was taken to verify the results. The mass spectrum for this compound required the addition of NaCl. The mass spectrum revealed a peak at 324.0842 amu. The chemical formula for 2 is $\text{C}_{16}\text{H}_{15}\text{NO}_3\text{Na}$, which gives a calculated molecular weight of 324.0848 amu. Several higher molecular mass peaks are also evident in the mass spectrum. These may arise from the impurity peaks seen in the NMR spectrum. Based on the $^1$H NMR, IR, and mass spectral data, it was concluded that 2 was successfully synthesized.

**Figure 12:** $^1$H NMR of 2
Figure 13: IR of 2
Figure 14: Mass Spectrum of 2
Synthesis of 3

Compound 3 strongly resembles the lead compound designed by Ronak Tilvawala in 2014. This structural analog primarily differs from that of L2 due to the absence of the ammonium group on the hydroxamic acid side chain.

The synthesis of C1 was based on the general procedure for the preparation of sodium salts of hydroxyalkanoic acids by Marvel and Birkhimer.\textsuperscript{25} δ-Valerolactone and potassium hydroxide were dissolved in water and heated for three hours at 70°C. The resulting salt was evaporated to dryness. The crude product was recrystallized from 95% ethanol and dried on the oil pump overnight to yield C1.

\textsuperscript{1}H NMR in D\textsubscript{2}O confirmed the synthesis of C1. A comparison between the spectrum of δ-valerolactone and C1 indicates many similarities; they differ, however, due to the downfield shift caused by the cyclization of the valerolactone ring. Due to the similar structures of these compounds, they maintain the same multiplicities. δ-Valerolactone presents peaks at 2.15 ppm (four protons), 2.55 ppm, and 4.34 ppm.\textsuperscript{26} C1 presents with the expected upfield shift in peaks at 1.408 ppm (four protons), 2.065 ppm, and 3.449 ppm for each of the methylene protons.

![Scheme 12: Synthesis of C2](image)
As shown in Scheme 12, C1 was then reacted with 4-methoxybenzyl chloride and sodium iodide in DMF and stirred at room temperature for 24 hours, as outlined by the procedure of Baldwin et. al.\textsuperscript{27}

An initial \(^1\)H NMR in DMSO did not support the synthesis of C2 due to significant impurities in the sample. A thin layer chromatography plate was run on the resulting crude reaction mixture in 70:30 hexanes:ethyl acetate showing that there were seven compounds within the crude product. The crude product was therefore purified by flash column chromatography in 70:30 hexanes:ethyl acetate to yield C2.

\(^1\)H NMR in DMSO indicated the diagnostic benzylic hydrogen singlet at 5.04 ppm indicating the successful addition of the methoxybenzyl group onto C1. Additionally, the two anticipated doublets at 7.00 ppm and 7.36 ppm to indicate the presence of a para-substituted aromatic group, which would be expected for the methoxybenzyl ester. The methoxybenzyl addition was also evidenced by the appearance of a singlet peak at 3.77 ppm with an integration of three protons, indicating the presence of a methyl group. The anticipated methylene peaks previously seen in C1, have separated into individual multiplet peaks at 1.41 ppm, 1.56 ppm, 2.31 ppm, 3.37 ppm, likely due to the decrease in electron density caused by the methoxybenzyl addition. Therefore the synthesis of C2 was confirmed.

\[\text{Scheme 13: Attempted synthesis pathway of C5}\]
In an attempt to synthesize C5, C2 was added to a pre-cooled triphosgene solution in DCM under nitrogen atmosphere at -20°C in an ethanol and ethylene glycol bath with dry ice for 10 minutes. Triethylamine was then added and the reaction mixture was stirred for 4 hours.

The product was analyzed using ATR IR spectroscopy. The IR spectrum of C5 was expected to have an acid chloride C=O stretch between 1770 - 1850 cm⁻¹ and an ester C=O stretch between 1715 - 1765 cm⁻¹ according to the structure shown above in Scheme 13. The spectral data indicated only the ester C=O stretch; it did not, however, contain a second C=O absorption band. It was therefore decided that the synthesis of C5 using this method was unsuccessful.

![Scheme 14: Synthesis of C3](image)

In order to avoid the synthesis of C5, we utilized the Stachulski method for synthesis of N-alkoxycarbonyl hydroxylamines, as shown above in Scheme 14. 1,1-Carboxyldiimidazole was added to C2 in acetonitrile and stirred at room temperature for 90 minutes. Imidazole and hydroxylamine hydrochloride were then added and stirred the mixture for 4 hours to completion to yield C3.

^1H NMR in DMSO was used to confirm the synthesis of C3. The diagnostic peaks that differed substantially between C2 and C3 include the amide singlet at 8.62 ppm and the hydroxyl
singlet at 9.51 ppm. Moreover, the presence of the methoxybenzyl is evident due to the benzylic protons at 4.98 ppm, as well as the doublets at 6.92 ppm and 7.28 ppm, which indicated a para-substituted aromatic ring. As discussed with respect to C2, the anticipated methylene hydrogens at 1.54 ppm, 2.31 ppm, and 4.38 ppm, and methyl proton at 3.73 ppm remained in the spectrum with minimal downfield shifting caused by the addition of the hydroxamide. Due to the reasonable purity of the product based on the ¹H NMR spectrum, the obtained product was used for the next step without further purification.

C4 was achieved by reacting C3 with phenyl chloroformate as described above for the synthesis of 1 from A2. The crude product was purified by chromatography on silica gel (90:10 hexane:ethyl acetate), yielding the product C4 as a colorless oil. The successful synthesis of C4 was confirmed by ¹H NMR.

The ¹H NMR spectrum of C4 differed from that of C3 due to the absence of the hydroxyl singlet at 9.51 ppm. Moreover, the spectrum of C4 contained a large multiplet at 3.51 ppm, rather than two distinct doublets, with an integration of nine protons, indicating the successful addition of the phenyl group. As is anticipated with O-acyl hydroxamates, the amide hydrogen at 8.62 ppm in the spectrum of C3 has been shifted downfield to 11.54 ppm, indicating the successful synthesis of C4.

TFA was then added to C4 in DCM following the Tilvawala procedure in order to remove the methoxybenzyl group to yield 3 as a white solid. The synthesis of 3 was confirmed by ¹H NMR in DMSO. As the TFA was expected to remove the methoxybenzyl group, the ¹H NMR displayed the absence of the benzylic proton at 4.98 ppm, as discussed for C3, as well as the four aromatic protons on the para-substituted aromatic of the methoxybenzyl group.
Moreover, the methylene peaks at 1.57 ppm, 2.23 ppm, and 4.12 ppm as well as the amide -NH peak at 11.55 ppm remained unchanged, as anticipated. A multiplet at 7.35 ppm integrating for five protons indicated the presence of a mono-substituted aromatic group.

To confirm the formation of 3, an IR spectrum (Figure 16) was taken and indicated the anticipated carbonyl peak at 1794.31 cm⁻¹. Finally, a TOF ESI⁺ mass spectrum was taken to verify the results (Figure 17). The mass spectrum for this compound also required the addition of NaCl. The mass spectrum revealed a peak at 320.0739 amu. The sodium ion adduct of 3 has a molecular formula of C₁₃H₁₅NO₇Na which gives a calculated molecular weight of 320.0746 amu. Based on the ¹H NMR, IR, and mass spectral data, it was concluded that 3 was successfully synthesized.
Figure 15: $^1$H NMR of 3
Figure 16: IR of 3
Figure 17: Mass Spectrum of 3
Synthesis of 4

Scheme 15: Synthesis of D1

The procedure for the synthesis of D1 was based on the procedure for Boc protection.\textsuperscript{29} 1-Amino-4-butanol was reacted with triethylamine and di-tert-butyl dicarbonate, as shown in Scheme 15. An initial $^1\text{H}$ NMR spectrum of the crude product indicated significant impurities in the aliphatic region of the spectrum. It was determined that the impurity was likely a butanol derivative based on the peaks at 1.5 ppm, 2.71 ppm, and 3.8 ppm with a possible -OH triplet at 4.64 ppm. Thus the crude product was purified by fractional distillation using the Kugelrohr apparatus in order to avoid decomposition on a silica column. The purified product was a yellow oil.

In order to confirm the presence of D1, a $^1\text{H}$ NMR spectrum was taken. The spectrum of 1-amino-4-butanol contains a quartet with an integration of four at 1.5 ppm, a triplet at 2.7 ppm integrating for two protons, and a triplet at 3.8 integrating for 2 protons. The amino group NH$_2$ has a shift of 7.3 ppm and the hydroxyl OH has a shift of 4.5 ppm.

On comparing the spectrum of the starting material to that of the product, it was evident that the methylene protons were shifted slightly due to the addition of the tert-butoxycarbonyl group, creating two distinct quartets at 2.87 ppm and 3.35 ppm for the methylene protons. The primary difference between the two spectra, however, was the appearance of a large singlet at
1.35 that integrated for nine protons. This peak represents the tert-butyl hydrogens as specified by the literature, which suggests the tert-butyl protecting group has a chemical shift of approximately 1.4 ppm.\textsuperscript{30} A one proton triplet at 6.75 ppm suggested an amide -NH. Therefore, it was determined that D1 was successfully synthesized and was used for the next step of the synthesis.

Much as with the synthesis of C3, D2 was synthesized by reacting D1 with 1,1’-carbonyldiimidazole and hydroxylamine hydrochloride. In order to determine if the hydroxamic acid was successfully formed from D1, a $^1$H NMR spectrum in DMSO was obtained. As anticipated, the Boc group and methylene protons remained mostly unaffected by the addition. However, the spectrum indicated the absence of triplet peak at 4.34 ppm indicative of the hydroxyl peak in D1, indicating that the starting material had been successfully reacted with the 1,1-carbonyldiimidazole and hydroxylamine hydrochloride. Rather, the spectrum indicated that as with the other hydroxamic acid products, the spectrum of the product D2 contained singlet peaks at 8.62 ppm for the amide hydrogen and at 9.48 ppm for the hydroxyl hydrogen. The spectrum also contained a triplet at 6.76 ppm that was assigned to the NH proton of the carbamate, which had remained mostly unchanged. Due to its high level of purity, D2 was used for the next step without further purification.

D2 was reacted with a solution of phenyl chloroformate in DCM as previously described. Initial spectra of the crude product indicated significant impurities in the final compound. The crude product was thus dissolved in ethyl acetate and was run on a TLC plate in an 80:20 hexane:ethyl acetate solvent system indicating three impurities. The primary impurity was starting material, D1 as evident from the hydroxamic acid hydroxyl and amide peaks in the crude
mixture spectrum. The other compounds were thought to be phenol and derivatives of the phenyl chloroformate as there were significant impurities in the aromatic region of the ¹H NMR spectrum.

The crude product was purified by flash column chromatography on silica gel from 80:20 hexane:ethyl acetate, and the fractions containing D₃ were evaporated to yield a yellow oil. The presence of D₃ was confirmed using ¹H NMR spectroscopy. The addition of the phenyl group was confirmed by the appearance of peaks in the aromatic region indicative of a monosubstituted aromatic ring. Moreover, as is typical in the formation of O-acyl hydroxamates, the amide proton shifted downfield to 11.53 ppm.²⁴

These findings were corroborated with ATR IR. As is the case for O-acyl hydroxamates, the peak at 1804 cm⁻¹ indicated the presence of the acyl hydroxamate C=O carbonyl bond. Moreover, the stretches at 3210.70 cm⁻¹ and 3359.50 cm⁻¹ suggested the presence of two secondary -NH bonds for the carbamate and amide respectively.

TFA was added to a solution of D₃ in DCM in order to remove the Boc protecting group to yield 4 as a yellow oil. The synthesis of 4 was confirmed by ¹H NMR in DMSO. In removing the protecting group, the neighboring amine nitrogen became protonated to yield an NH₃⁺. This was corroborated by the spectrum, which contained a broad singlet at 7.66 ppm with an integration of three. As anticipated, the methylene protons at 1.64 ppm, 2.49 ppm, and 4.18 ppm, as described in the synthesis of D₁, remained largely unchanged. Similarly, the phenyl group at 7.32 ppm indicated the anticipated five protons and remained similar to that of D₃. As discussed with D₃, the O-acyl hydroxamate NH singlet at 11.58 remained unchanged.
To further support the formation of 4, an IR spectrum was taken as shown in Figure 19. As anticipated, the final IR spectrum contained the typical O-acyl hydroxamate carbonyl stretch previously discussed. Moreover, the spectrum contained three broad peaks at approximately 3000 cm\(^{-1}\), 3160 cm\(^{-1}\), and 3300 cm\(^{-1}\). This range is likely indicative of the amide and ammonium N-H bonds. As ammonium typically produces three bands in this region and the secondary amide would produce one band, it is likely that the bands have significant overlap causing them to appear as three broad peaks. A TOF ESI\(^+\) mass spectrum was taken to verify the results as shown in Figure 20. The mass spectrum revealed a peak at 269.1136 amu. The chemical formula for 4 is C\(_{12}\)H\(_{17}\)N\(_2\)O\(_3\), which gives a calculated molecular weight of 269.1137 amu. Based on the \(^1\)H NMR, IR, and mass spectral data, it was concluded that 4 was successfully synthesized.

![Figure 18: \(^1\)H NMR spectrum of 4](image-url)
Figure 19: ATR IR of 4
Figure 20: Mass Spectrum of 4
Spontaneous Hydrolysis of 1-4 in Aqueous Solution

The spontaneous hydrolysis of compounds 1, 2, 3, and 4 in aqueous solution was studied in order to evaluate their comparative rates of reaction with the P99 β-lactamase. The pseudo-first order rate constant for the hydrolysis of compounds 1, 3, and 4 was obtained by fitting the spectrophotometric data to an exponential function in Kaleidagraph.

Reaction of 1 in aqueous solution

To determine the rate of spontaneous hydrolysis of 1 in aqueous solution, 1 was dissolved in DMF to obtain a 10 mM stock solution and 5 μL of the stock was added to 20 mM MOPS buffer, pH = 7.5. The UV absorption spectral change was followed at $\lambda = 270$ nm for 3600 seconds. The overlay of the initial and final absorption spectra is shown in Figure 21. The absorption at 270 nm as a function of time was plotted in Kaleidagraph and the hydrolysis rate constant was determined to be $4.04 \pm 0.06 \times 10^{-4}$ s$^{-1}$. The fitted progress curve of the spontaneous hydrolysis is shown in Figure 22.

![Initial and final absorption spectra overlay of 1](image)

**Figure 21:** Initial and final absorption spectra overlay of 1
Figure 22: Spontaneous Hydrolysis of 1
Reaction of 2 in aqueous solution

To determine the rate of spontaneous hydrolysis of 2 in aqueous solution, 5 microliters of 10 mM 2 in DMF was added to 20 mM MOPS buffer, pH = 7.5. The overlaid UV/Vis absorption spectra of the initial and final absorption spectra showed no significant change over time. Therefore, it was not possible to determine the rate of spontaneous hydrolysis of 2 by this method.

Reaction of 3 in aqueous solution

The hydrolysis of 3 was studied spectrophotometrically as described for 1. The overlay of the initial and final absorption spectra are shown in Figure 23. The rate constant of hydrolysis absorption at 270 nm was plotted and fitted in Kaleidagraph and the rate constant of hydrolysis of 3 was determined to be 5.0 ± 0.4 x 10^-4 s^-1. The fitted progress curve of the spontaneous hydrolysis is shown in Figure 24.

![Absorption spectra overlay of 3](image)

**Figure 23:** Initial and final absorption spectra overlay of 3
**Figure 24**: Spontaneous hydrolysis of 3
Reaction of 4 in aqueous solution

The hydrolysis of 4 was studied spectrophotometrically as described for 1. The overlay of the initial and final absorption spectra are shown in Figure 25. The rate constant of hydrolysis was plotted in Kaleidagraph and the rate constant of hydrolysis of 4 was determined to be 4.7 ± 0.4 x 10⁻⁴ s⁻¹. The fitted progress curve of the spontaneous hydrolysis is shown in Figure 26.

Figure 25: Initial and final absorption spectra overlay of 4
Figure 26: Spontaneous hydrolysis of 4
Comparison of rate constants of spontaneous hydrolysis

The rate constants of spontaneous hydrolysis for compounds 1, 3, and 4 as well as lead compounds L1 and L2 are shown below in Table 1.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Rate Constant of Spontaneous Hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.04 x 10^{-4} s^{-1}</td>
</tr>
<tr>
<td>3</td>
<td>4.98 x 10^{-4} s^{-1}</td>
</tr>
<tr>
<td>4</td>
<td>4.67 x 10^{-4} s^{-1}</td>
</tr>
<tr>
<td>L1^{31}</td>
<td>2.5 x 10^{-4} s^{-1}</td>
</tr>
<tr>
<td>L2^{31}</td>
<td>1.7 x 10^{-4} s^{-1}</td>
</tr>
</tbody>
</table>

Table 1: Comparison of rate constants of spontaneous hydrolysis.

The spontaneous hydrolysis of a compound is dependent on the ability of the leaving group. A lower pKa indicates a better leaving group. As indicated by Scheme 16 below, O-aryloxyacarbonyl hydroxamates hydrolyze to form carbon dioxide, phenol, and a hydroxamic acid leaving group. The approximate pKa of an unsubstituted hydroxamic acid is 8.88 in water and that of phenol is 9.95.^{32} Thus hydroxamate should be the better leaving group and depart first from the carbonyl. Due to the presence of substituents on the hydroxamic acid, the chemical
reactivity of a O-aryloxycarbonyl hydroxamate is determined by the electronegativity of these substituents.

\[
\text{R-OCNHO\textsubscript{COOPh}} \xrightarrow{\text{H}_2\text{O}} \text{R-OCNHOH} + \text{CO}_2 + \text{PhOH}
\]

**Scheme 16**: Spontaneous hydrolysis of O-aryloxycarbonyl hydroxamate into hydroxamic acid leaving group, carbon dioxide, and phenol.
Inhibition of P99 β-lactamase

Compounds 1, 2, 3, and 4 were designed and synthesized as analogs of compound L1 and L2, where the effects of different elements of the structures of the latter compound were assessed separately. Their structures are shown in Table 2 below. Due to their structural similarities, it was assumed that like L1 and L2, compounds 1, 2, 3, and 4 were probably not substrates of P99 β-lactamase. Thus, we proceeded with the time dependent inhibition experiments.\textsuperscript{31,21}

<table>
<thead>
<tr>
<th>Compound</th>
<th>Hydroxamic Acid Side Chain</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>![Image of Compound 1]</td>
</tr>
<tr>
<td>2</td>
<td>![Image of Compound 2]</td>
</tr>
<tr>
<td>3</td>
<td>![Image of Compound 3]</td>
</tr>
<tr>
<td>4</td>
<td>![Image of Compound 4]</td>
</tr>
<tr>
<td>L1</td>
<td>![Image of Compound L1]</td>
</tr>
</tbody>
</table>
Table 2: Comparison of hydroxamic acid side chains of inhibitors. R = OCOOPh

Reaction of P99 β-lactamase with 1

The reaction between 1 and the P99 β-lactamase enzyme was initially analyzed by following the inactivation of P99 β-lactamase enzyme at various concentrations of 1 after the inactivation reactions had gone to completion (t = 10 hours). Nine mixtures containing 150 nM P99 enzyme and the following concentrations of 1: 0.00 μM, 0.01 μM, 0.02 μM, 0.05 μM, 0.10 μM, 0.15 μM, 0.20 μM, 0.50 μM, and 1.0 μM were incubated at 25 °C. Aliquots of the (20 μL) of the reaction mixture were added to the substrate, cephalothin. The hydrolysis of the β-lactam ring on the substrate cephalothin produces a UV spectral change which was monitored at λ = 270 nm. For each assay the initial rate of the reaction was plotted against time for each of the respective inhibitor concentration shown in Figure 27.

Increased concentrations of 1 generally resulted in greater inhibition of enzymatic function; however, the final activity of the enzyme may not go to zero, despite the inhibitor concentration being 6.7 times the enzyme concentration. This indicates that turnover or spontaneous hydrolysis may occur as well as inhibition. An approximate line of best fit was drawn through the initial points, we calculated that close to one molecule of 1 are required to
inactivate one molecule of P99 enzyme. Therefore, 1 did not experience a significant rate of turnover.

**Figure 27:** Concentration-based loss of enzymatic activity of P99 (0.15 μM) in the presence of 1.
To further test the loss of enzymatic activity of P99 in the presence of 1, time-based loss of enzyme activity assays were analyzed. P99 enzyme (150 nM) was incubated with 1000 nM 1. Aliquots (20 μL) of the reaction mixture were added to the substrate cephalothin and the reaction was monitored at at $\lambda = 270$ nm using UV absorption spectroscopy. The initial rate versus time data was plotted and the best fit to the data was determined using the Dynafit program, and where $k_1$ represents the rate constant for inhibition and $k_0$ represents spontaneous hydrolysis. The data and fits to Scheme 8, as shown in Figure 28.

$$\begin{align*} E + I & \xrightarrow{k_1} EI \\ I & \xrightarrow{k_0} P \end{align*}$$

Scheme 8

In order to further test the time-based inactivation of enzymatic activity of P99 in the presence of 1, 150 nM P99 enzyme was incubated with 500 nM 1. As with the initial time-based inactivation, the initial rate of reaction versus time data was plotted and the best fit of the data to Scheme 8 was determined using the Dynafit program, shown in Figure 29. The rate of spontaneous hydrolysis, $k_0$, was fixed at $4.04 \times 10^{-4} \text{ s}^{-1}$. The fits shown in Figures 28 and 29 were simultaneous to both experiments to give a value for $k_1$ determined to be $5030 \pm 120 \text{ M}^{-1} \text{s}^{-1}$.

In neither time-dependent inactivation experiment was a return in enzymatic activity observed. Therefore, the inhibition of P99 by 1 was determined to be irreversible, as was that by L1 and L2.
Figure 28: Timed inhibition of 150 nM P99 with 1000 nM 1

Figure 29: Timed inhibition of 150 nM P99 with 500 nM 1
**Reaction of P99 β-lactamase with 2**

Despite unsuccessful attempts to obtain a rate of spontaneous hydrolysis for 2, the concentration-based loss of enzymatic activity experiment at various concentrations of 2 was conducted. Activity measurements were made after the inactivation reactions had gone to completion (t = 14 hours). Eight assays at concentrations of 2 were incubated with 150 nM P99 enzyme: 0.00 μM, 0.01 μM, 0.02 μM, 0.05 μM, 0.10 μM, 0.20 μM, 0.50 μM, and 1.0 μM. Aliquots (20 μL) of the reaction mixture were added to the cephalothin substrate and the hydrolysis of the cephalothin was monitored at λ = 270 nm using UV absorption spectroscopy. For each assay the initial rate of the reaction was plotted against the respective inhibitor concentration, as shown in Figure 30. As indicated by Figure 30, increased concentrations of 2 resulted in greater inhibition of enzymatic function and the final activity of the enzyme went to zero, indicating that 2 is an inhibitor of P99 β-lactamase. An approximate line of best fit was plotted and it was approximated that one molecule of 2 is required to inactivate one molecule of P99 enzyme.
Figure 30: Concentration-based loss of enzymatic activity of P99 (0.15 μM) in the presence of 2.
To further test time-based loss of enzymatic activity of P99 in the presence of 2, 150 nM P99 enzyme was incubated separately with 1000 nM and 500 nM 2. Aliquots (20 µL) of the reaction mixture were added to the substrate cephalothin and the reaction was monitored at at λ = 270 nm using UV absorption spectroscopy. The initial rates were taken at various times during the reaction. The initial rate data was plotted against time and the best fit of the data to Scheme 8 determined from the Dynafit program and is shown in Figure 31 for 1000 nM 2 and Figure 32 for 500 nM 2. In neither time-dependent inactivation experiment was a return in enzymatic activity observed. Therefore, the inhibition of P99 by 2 was determined to be irreversible.

Because no spontaneous hydrolysis of 2 could be detected spectrophotometrically, the rate constant for the spontaneous hydrolysis of 1 was used as an approximation based on the structural similarities between the molecules and lack of polar groups on the substituent. Therefore $k_0$ was fixed at $4.04 \times 10^{-4}$ s$^{-1}$. The value for $k_1$ was determined to be $52600 \pm 5900$ M$^{-1}$ s$^{-1}$. 
Figure 31: Timed inhibition of 150 nM P99 with 1000 nM 2

Figure 32: Timed inhibition of 150 nM P99 with 500 nM 2
Reaction of P99 β-lactamase with 3

Loss of enzymatic activity studies were conducted to analyze the inhibitory power of 3. Concentration-based loss of enzymatic activity experiments at various concentrations of 3 were conducted and the initial rates were determined after the inactivation reactions had gone to completion (t = 9 hours). Eight concentrations of 3 were incubated with 150 nM P99 enzyme: 0.00 μM, 0.01 μM, 0.02 μM, 0.05 μM, 0.10 μM, 0.20 μM, 0.50 μM, and 1.0 μM. Aliquots (20 μL) of the reaction mixture were added to the cephalothin substrate and the hydrolysis of the cephalothin was monitored at λ = 270 nm using UV absorption spectroscopy. For each assay, the initial rate of the reaction was plotted against the respective inhibitor concentration, as shown in Figure 33.

As with 2, increasing concentrations of 3 resulted in greater inhibition of enzymatic function indicating that 3 is an inhibitor of P99 β-lactamase. An approximate line of best fit was plotted and it seemed likely that one molecule of 3 is required to inactivate one molecule of P99 enzyme.
Figure 33: Concentration-based loss of enzymatic activity of P99 (0.15 μM) in the presence of 3.
To further test time-based loss of enzymatic activity of P99 in the presence of 3, 150 nM P99 enzyme was incubated separately with 1000 nM and 500 nM 3. Aliquots (20 μL) of the reaction mixture were added to the substrate cephalothin and the reaction was monitored at \( \lambda = 270 \) nm using UV absorption spectroscopy. The initial rates were taken at various times during the reaction. The initial rate data was plotted against time and the best fit of the data to Scheme 8 determined from the Dynafit program and is shown in Figure 34 for 1000 nM 3 and Figure 35 for 500 nM 3. In neither time-dependent inactivation experiment was a return in enzymatic activity observed. Therefore, the inhibition of P99 by 3 was determined to be irreversible.

As previously discussed, the spontaneous hydrolysis for 3 was determined to be \( 4.98 \times 10^{-4} \) s\(^{-1}\). Therefore \( k_0 \) in Scheme 8 was fixed at \( 4.98 \times 10^{-4} \) s\(^{-1}\). Using the Dynafit program, the value for \( k_1 \) was determined to be \( 39500 \pm 3330 \) M\(^{-1} \) s\(^{-1}\).
Figure 34: Timed inhibition of 150 nM P99 with 1000 nM 3

Figure 35: Timed inhibition of 150 nM P99 with 500 nM 3
Reaction of P99 β-lactamase with 4

Loss of enzymatic activity studies were conducted to analyze the inhibitory power of 4. Concentration-based loss of enzymatic activity experiments at various concentrations of 4 were conducted and the initial rates were determined after the inactivation reactions had gone to completion (t = 16 hours). Seven concentrations of 4 were incubated with 150 nM P99 enzyme: 0.00 μM, 0.01 μM, 0.02 μM, 0.05 μM, 0.10 μM, 0.20 μM, 0.50 μM, and 1.0 μM. Aliquots (20 μL) of the reaction mixture were added to the cephalothin substrate and the hydrolysis of the cephalothin was monitored at λ = 270 nm using UV absorption spectroscopy. For each assay, the initial rate of the reaction was plotted against the respective inhibitor concentration, as shown in Figure 36.

Increased concentrations of 4 generally resulted in greater inhibition of enzymatic function, indicating that 4 inhibits P99 β-lactamase. An approximate line of best fit was drawn through the initial points, and it seemed likely that one molecule of 4 is required to inactivate one molecule of P99 enzyme.
Figure 36: Concentration-based loss of enzymatic activity of P99 (0.15 μM) in the presence of 4.
To further test the loss of enzymatic activity of P99 in the presence of 4, time-based loss of enzyme activity assays were analyzed. 500 nM 4 and 1000 nM 4 were incubated with 150 nM P99 enzyme. Aliquots (20 μL) of the reaction mixture were added to the substrate cephalothin and the reaction was monitored at λ = 270 nm using UV absorption spectroscopy. The initial rate versus time data was plotted and the best fit of the data were determined using the Dynafit program using Scheme 8 due to the lack of measurable turnover.

The best fit curves of the data are shown below in Figure 37 for the 500 nM 4 and Figure 38 for the 1000 nM 4. The rate of spontaneous hydrolysis, $k_o$, was fixed at $4.67 \times 10^{-4}$ s$^{-1}$. The value for $k_i$ was determined to be $15500 \pm 2800$ M$^{-1}$ s$^{-1}$.

Figure 37: Timed inhibition of 150 nM P99 with 1000 nM 4
Figure 38: Timed inhibition of 150 nM P99 with 500 nM 4
Comparison of Rate Constants from Inhibition Experiments

<table>
<thead>
<tr>
<th>Compound</th>
<th>$k_1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5030 ± 120 M$^{-1}$ s$^{-1}$</td>
</tr>
<tr>
<td>2</td>
<td>52600 ± 5900 M$^{-1}$ s$^{-1}$</td>
</tr>
<tr>
<td>3</td>
<td>39500 ± 3300 M$^{-1}$ s$^{-1}$</td>
</tr>
<tr>
<td>4</td>
<td>15500 ± 2800 M$^{-1}$ s$^{-1}$</td>
</tr>
<tr>
<td>L1$^{31}$</td>
<td>6100 ± 200 M$^{-1}$ s$^{-1}$</td>
</tr>
<tr>
<td>L2$^{21}$</td>
<td>35000 ± 400 M$^{-1}$ s$^{-1}$</td>
</tr>
</tbody>
</table>

Table 3: Summary of rate constants from inhibition experiments.

The table above summarizes the experimental findings of the loss of enzymatic activity experiments for compounds 1, 2, 3, and 4. The rate constants from these experiments can be compared to each other as well as to those of L1 and L2 in order to understand the reactivity of these compounds with P99 β-lactamase.

Comparing the inactivation rate constants, $k_1$, of 1 and L1, it appears that both compounds are similarly reactive as inhibitors of P99 β-lactamase; L1, however, is slightly more reactive than 1 based on its larger rate constant. This difference in rate constants can be
attributed to the structural differences between the two compounds. The more rigid phenyl ring probably fits more stably into the active site.

Moreover, based on the rate constants, **L2** is substantially more reactive as an inhibitor of P99 than **1**. This difference is likely caused by the interaction of the polar termini of **L2**, which interact with specific residues in the Ω loop of the active site, leading to greater stability of the acyl-enzyme complex, as shown in the modeled structure of **L2** (Figure 39). Therefore, **L2** produces a more favorable interaction with the enzyme than the non-polar butyl group on **1**, leading to higher activity.

![Figure 39: Interaction of polar termini of **L2** tetrahedral intermediate with Ω loop of active site.](image)

A comparison of the rate constants of **2** and **1** indicate that **2** is a stronger inhibitor than **1**. Due to the favorable interaction of the phenyl group with the active site seen with compound **L1**,
this result was anticipated. However, it is interesting to note that 2 has a rate constant that is nearly an order of magnitude larger than that of L1. This is likely due to the addition of the methylene group. This additional methylene group would move the phenyl substituent 1.54 Å, where it must fit better into the active site.

Comparing 3 and 4 indicates that 3 is more reactive as an inhibitor than 4. It can therefore be assumed that the carboxylate has a substantially more favorable interaction with the enzyme than that of the ammonium. This may be a result of the interactions of the charges within the Ω loop of the active site. However, when comparing 3 and 4 to L2, which contains both the carboxyl and ammonium groups, L2 is more reactive than 4, but slightly less reactive than 3, indicating that the Arginine 204 has a more stabilizing interaction with the tetrahedral intermediate than the Serine 212.
Synthesis of 5

![Chemical structure of compound 5](image)

**Figure 40**: Proposed inhibitor 5

Due to the success of the inhibitor 2, additional inhibitor designs will likely contain an aromatic group that allows for the preferred interactions with the enzyme active site. Moreover, due the high reactivity of L2, it seems important to retain both the carboxylate and ammonium moieties. These considerations lead directly to compound 5 (Figure 40). The synthesis has been underway. The synthesis of 5 follows the synthesis pathway shown below in Scheme 18. The procedures for this synthesis can be found in Appendix 1. At the present, we have achieved synthesis of the E3 intermediate on a small scale.
Scheme 17: Synthesis of 5
Conclusions and Future Work
In this thesis, the synthesis of compounds 1, 2, 3, and 4 have been described. Their capabilities as inhibitors of *Enterobacter cloacae* P99 β-lactamase were evaluated through kinetic studies. Through concentration-dependent and time-dependent loss of enzymatic activity assays, it has been shown that 1, 2, 3, and 4 irreversibly inhibit P99 β-lactamase. Given the irreversible inhibition of these compounds, it is likely that they follow similar pathways to those established for this class of compounds.\(^{21}\)

Based on the rate constants of \(k_i\), it is likely that 1 inhibits through the same mechanism as \(L_1\), while 2, 3, and 4 inhibit through the same mechanism as \(L_2\) (see these mechanisms in the introduction). Mass spectrometric analysis of the inhibited acyl-enzyme complexes will allow for definitive findings.

Of the four compounds, compound 2 was the most effective inhibitor against P99 β-lactamase. Moreover, 2 was a more effective inhibitor than \(L_2\). This indicates that the addition of the phenyl group onto the hydroxamic acid of \(L_2\) may lead to greater inhibitory properties.

In order to continue this work, compound 5 will be used to examine the properties of the addition of ammonium and carboxylate substituents onto a phenyl ring para to the O-acyl hydroxamate. Additional substituent groups may be looked at for possible inhibitors based on the results of the concentration-dependent and time-dependent loss of enzymatic activity assays of 5. O-acyl hydroxamates retain high promise as inhibitors of class C β-lactamases.
References


17, 2016).


Appendices
Appendix 1

Synthesis of 5

Intermediate 1 (E1): To a pre-cooled solution of t-butyl D-tyrosine (5.00 g, 21.0 mmol) and triethylamine (3.13 g, 31 mmol) in 66% aqueous dioxane (90 mL), a solution of di-tert-butyl dicarbonate (5.04 g, 23.1 mmol) in dioxane (30 mL) was added and the mixture was stirred at room temperature for 4 hours. The solvent was removed by rotary evaporation and the resulting residue was partitioned between ethyl acetate and 10% citric acid. The product was extracted from the aqueous layer with ethyl acetate three times and the combined organic layer was washed with a 5% sodium bicarbonate solution and dried over magnesium sulfate. Excess solvent was removed via rotary evaporation, yielding the product E1 as a yellow oil (4.01 g, 14.2 mmol, 67.5 % yield). The obtained product was used for the next step without further purification.

Intermediate 2 (E2): To E1 (2.0 g, 7.1 mmol) in acetonitrile (10 mL), 1,1’-carbonyldiimidazole (1.76 g, 10.9 mmol) was added and stirred at room temperature for 90 minutes. To the reaction mixture, imidazole (1.47 g, 25.9 mmol) and O-benzylhydroxylamine hydrochloride (1.88 g, 10.9 mmol) were added and stirred for 4 hours to completion. The imidazole hydrochloride precipitate was filtered out of the reaction mixture and the filtrate was partitioned between ethyl acetate and 0.2 M citric acid. The organic layer was washed with citric acid, dried over sodium sulfate, and evaporated to dryness to obtain E2. Yield: (3.00 g, 5.76 mmol, 81.1 % yield) The obtained product was used for the next step without further purification.
Intermediate 3 (E3): Compound E2 (0.50 g, 0.96 mmol) was dissolved in ethanol (5 mL). Pd/C (0.13 g, 0.095 mmol) catalyst was added and the reaction was hydrogenated at 45 psi for 4.5 hours. The catalyst was filtered off, the filtrate was concentrated using rotary evaporation, and the residue was dried overnight on the oil pump. The residue was then washed with ethyl acetate and the precipitate was dried to obtain E3 as a white solid. Yield: (0.28 g, 0.61 mmol, 63.5 % yield).