Synthesis and Analysis of Novel Mandelamide Hydrolase Substrates

by

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Abstract

The amidase signature (AS) enzyme superfamily is a widespread family of enzymes that catalyze the hydrolysis of an amide bond. AS enzymes are present in almost all organisms, and operate on a wide variety of substrates. The superfamily is defined not only by the common family of molecules hydrolyzed, but also by a large stretch of sequence homology in the genes that code for the enzymes. Recently mandelamide hydrolase (MAH) has been isolated and characterized as a member of the AS superfamily. The specificity and mechanism of action of MAH are not completely understood. Additionally, it has been suggested that AS enzymes operate through a similar mechanism to that of β-lactam recognizing enzymes (BLREs) due to the high level of similarity in active site structure between the two families. Here the synthesis of a series of O-carbamoyl hydroxamates as well as a series of p-nitriphenyl carbonates is examined and a kinetic evaluation of the interactions between these molecules and MAH is presented. It is shown that MAH requires either a small amine leaving group, or a good phenol leaving group to complete hydrolysis. This indicates that there are two distinct portions of the MAH active site; one that binds the amide, and is small and specific, and a larger more general aromatic binding site. It has previously been shown that molecules similar to those investigated here are capable of crosslinking the BLRE active site, whereas here it is shown that O-carbamoyl hydroxamates are simply substrates of MAH. This indicates that while the active site functional groups of the two families of enzymes are similar, major differences in active site structure still exist. This signifies that while the
general mechanisms employed by the two families of enzymes may be similar, different modes of inhibition may be found for each family.

1. Introduction

1.1 Amidase Signature Enzyme Superfamily

The amidase signature (AS) enzyme superfamily is a broad family of enzymes that have large amounts of sequence homology over a stretch of greater than 150 amino acid residues that is rich in serine and glycine, and catalyze the hydrolysis of an amide bond\(^1\). The homologous stretch is called the amidase signature sequence, and over 100 different enzymes are known to contain the sequence\(^1\). These enzymes cover a wide variety of organisms and functions, such as fatty acid amide hydrolase (FAAH), found in humans, which hydrolyzes sleep inducing lipids and cannabinoids\(^2\). Other substrates of the AS family include polypeptide chains and smaller amides such as malonamide and mandelamide (Figure 1.1). The sequence homology defining the family thus indicates a similarity in catalytic residues, and not active site shape of the various enzymes in the family.
Figure 1.1: Four natural substrates of AS enzymes are shown: A, anandamide, and B, oleamide are substrates of FAAH; C shows a peptide chain, the substrate of peptide amidase (PAM), and D, mandelamide, the substrate of mandelamide hydrolase (MAH).

The catalytic residues of the AS family are well characterized and are conserved throughout all of the structures that have been solved thus far. The residues exist in a Ser-cis-Ser-Lys triad as shown in figure 1.2 below.

Figure 1.2: The catalytic triad of FAAH is shown with bound methoxyarachidonyl phosphonate (MAP).
While the catalytic residues are highly conserved, there are two different proposed mechanisms in the literature for the AS family. The general difference between the two mechanisms is whether or not the catalytic lysine is protonated in the active site of the enzyme. In the mechanism for FAAH (figure 1.3), the lysine is deprotonated and serves as a general base\(^1\), whereas in the mechanism for PAM (figure 1.4) the lysine is protonated and serves as a general acid\(^3\). In both mechanisms one serine acts as a nucleophile, while the other serine acts as a proton transfer mediator\(^1\).

**Figure 1.3:** The mechanism for FAAH is shown. Note the catalytic lysine is deprotonated.
Figure 1.4: The mechanism for PAM is shown. Note that the catalytic lysine is protonated.

The existence of two different mechanisms for different AS enzymes has led to a suggestion that there are two classes of enzymes within the family that employ the two different mechanisms of catalysis\(^3\). But, in both of the proposed mechanisms hydrolysis occurs through the presence of a covalent acyl-enzyme. This indicates that one potential method of inhibition could involve the formation of an inert acyl-enzyme adduct.
1.2 The Mandelate Pathway

The mandelate pathway is possessed by a wide variety of soil and water dwelling microorganisms and allows for bacteria to utilize mandelate as a primary source of carbon, and thus energy production. Mandelate is a product of plant origin that exists naturally as both the D and L enantiomers and is the initial input in most organisms that utilize the pathway\(^4\). Mandelate induces the production of mandelate dehydrogenase, benzoylformate decarboxylase, and benzaldehyde dehydrogenase which serve to catalyze the conversion of mandelate into benzoate. The presence of benzoate serves to repress the expression of mandelate dehydrogenase, benzoylformate decarboxylase, and benzaldehyde dehydrogenase, so the enzymes in the pathway are only produced in the presence of starting material. Benzoate also serves to induce expression of enzymes farther along in the pathway which convert it into catechol. Catechol is then converted to \(\beta\)-ketoadipate, which can be broken down into succinyl-coA and acetyl-coA\(^5\).

![Chemical diagram of the mandelate pathway](image)

**Figure 1.5:** The mandelate pathway, with an input of mandelatic acid and an output of succinyl CoA.
Most organisms that utilize the mandelate pathway are only capable of using one enantiomer of mandelate as a source of carbon, but *Pseudomonas putida* is an exception\(^6\). *P. putida* possesses two additional enzymes that are used in the mandelate pathway, mandelamide hydrolase and mandelate racemase. Mandelamide hydrolase catalyzes the hydrolysis of the amide bond of both the D and L enantiomers of mandelamide to produce mandelate and ammonia. Mandelate racemase converts D-mandelate into L-mandelate, and allows for *P. putida* to use both enantiomers in the mandelate pathway\(^7\) (Figure 1.6). The presence of these two enzymes allows for *P. putida* to utilize the mandelate pathway for a wider set of substrates than other microorganisms.

**Figure 1.6:** The two additional steps in the mandelate pathway in *P. putida*: A, the hydrolysis of mandelamide to mandelic acid by MAH; B, the conversion of D-mandelic acid to L-mandelic acid by mandelate racemase.

### 1.3 MAH Structure/Specificity

MAH catalyzes the conversion of mandelamide to mandelate through the hydrolysis of the amide bond. While the crystal structure of MAH has not been
solved, homology studies and analysis of the amino acid sequence for MAH have determined the catalytically active residues to be Ser180, Ser204, and Lys 100. Enzymes with mutations to the three proposed catalytic residues have been prepared and tested, and it has been shown that mutations to Ser204 and Lys100 lead to a loss of all catalytic activity. This is consistent with similar experimentation on malonamidase E2 (MAE2) and PAM, while in FAAH the equivalent mutation to the catalytic lysine residue produces an enzyme with extremely reduced (<0.005%) catalytic activity. Mutation to Ser180 has been shown to result in an enzyme that retains a small level of activity, which is consistent with experiments performed on MAE2, PAM and FAAH.

As shown in figures 1.3 and 1.4 there are two proposed mechanisms employed by AS enzymes. One indicator of this is the relative rate of hydrolysis of esters as compared to amides by enzymes within the family. Based on the fact that both FAAH and MAH hydrolyze esters at a similar rate as they do amides it has been suggested that they are in the same subfamily, and utilize an uncharged lysine residue in catalysis.

The substrate specificity of MAH has recently been examined, and it has been shown to have greater specificity than other AS enzymes. For example, FAAH has been shown to hydrolyze a wide variety of molecules reasonably well as shown in figure 7.
MAH exhibits a high level of substrate specificity, as hydrolysis of the amide bond does not occur when the nitrogen is bound to a large substituent. The presence of a methyl group on the nitrogen of the amide bond has been shown to decrease $k_{cat}/K_m$ values dramatically, and the presence of an ethyl group or larger has been shown to prevent hydrolysis of the amide bond entirely (Figure 1.8).  

The acyl side chain specificity has also been examined, and MAH displays a high level of specificity for aromatic substrates. Substitution of a cyclohexyl ring in place of a phenyl ring leads to a decrease in $k_{cat}/K_m$ by three orders of magnitude, indicating that the active site requires the rigid structure of an aromatic ring to properly bind the substrate. Additionally, substitution of linear hydrocarbon chains in place of a phenyl ring leads to a drop in $k_{cat}/K_m$ by three orders of magnitude for a
chain length of 5, and by 6 orders of magnitude when the chain length is 1 carbon\textsuperscript{10}. Interestingly, the $k_{\text{cat}}$ values for various substrates do not change dramatically; aromatic substrates, aliphatic rings and unbranched carbon chains all have similar turnover rates. Instead it is the $K_m$ values that change dramatically\textsuperscript{10}. This indicates that MAH has an active site that only binds specific substrates, but that the enzyme is quite good at catalyzing hydrolysis of an amide bond regardless of substrate structure.

1.4 Link to $\beta$-lactam Recognizing Enzymes

The catalytic triad of the AS superfamily closely resembles the catalytic tetrad present in $\beta$-lactam recognizing enzymes (BLRE), which consists of two lysines and two serines. Possible catalytic schemes are shown in figure 1.8, featuring a lysine acting as a general base, and a serine acting as a general acid in the formation of an acyl intermediate\textsuperscript{11}.

![Figure 1.9: Two mechanisms utilized by BLREs are shown.\textsuperscript{11}](image)

This is similar to the general schemes shown above in figures 1.3 and 1.4. When active sites from enzymes within both families are compared, more similarities
emerge. The difference in position between the catalytically active Ser1 O, Ser2 O, and Lys1 N in FAAH and the BLRE DD peptidase from *Actinomadura* R39 has been found to average 0.189 Å, and comparing the same three atoms to a variety of other BLRE enzymes produces an average difference in position of 0.27 Å ± 0.14 Å. This high level of similarity is shown in figure 1.10.

![Figure 1.10: Overlap of the active sites of BLREs (elemental colors) and AS enzymes (blue).](image)

The high level of similarity between the AS enzyme active sites and the BLRE enzyme active sites has been postulated to have occurred through convergent evolution. Due to the high level of similarity it is possible that molecules that inhibit one family of enzymes could serve as inhibitors for the other family.
1.5 Antibiotic Resistance

Penicillin was first used as an antibiotic agent about 70 years ago following its discovery by Alexander Fleming. It was the first β-lactam antibiotic to be used in the fight against infectious diseases, and much of the struggle against bacterial infections that occurs today deals with the increased resistance that bacteria have developed since penicillin was first used\textsuperscript{12}. Bacteria have shown a remarkable ability to develop antibiotic resistance on a short timescale. In 1941 nearly all strains of \textit{Staphylococcus aureus} were susceptible to attack by penicillin, but just three years later, in 1944, \textit{S. aureus} had developed or acquired enzymes capable of breaking down penicillin, referred to as β-lactamases. Today greater than 95% of all \textit{S. aureus} strains are resistant to penicillin, emphasizing the incredible ability of bacteria to adapt to their environment and develop resistance to potential dangers\textsuperscript{13}.

The development of penicillin resistance in bacteria led to the discovery of a wide variety of new β-lactam antibiotics such as cephalosporins and carbapenems.

\textbf{Figure 1.11:} Three families of β-lactam antibiotics are shown: A, carbapenems; B, penicillins; C, cephalosporins.

However, as with penicillins, bacteria developed new defenses rapidly to these new families of antibiotics as well. Additionally, bacteria readily transfer genes. This
ability allows them to essentially share their defenses, making it difficult to find effective long term antibiotics. The rise of infectious bacteria that are resistant to many different antibiotics, and thus are very difficult to kill, has become a major health threat\textsuperscript{13}. Methicillin-resistant \textit{S. aureus} (MRSA) is one example of a bacterium that displays high levels of resistance to many different types of antibiotics, making treatment difficult\textsuperscript{13}. This incredible ability of bacteria to adapt to their surroundings and threats to their survival is a dangerous one for humans.

1.6 BLRE Inhibitors

The prevalence of antibiotic resistance has led to an emphasis on developing inhibitors for BLREs, and thus many mechanisms of inhibition for these enzymes are well understood. One of the earliest $\beta$-lactamase inhibitors, clavulanic acid, inhibits $\beta$-lactamases through a covalent intermediate in which the enzyme recognizes the $\beta$-lactam ring in the inhibitor, and attacks the ring. This leads to an inert, inhibited acyl-enzyme\textsuperscript{14}.

Covalent inhibition is one method for rendering an enzyme inert, and commonly occurs through acylation of the enzyme. O-Aryloxycarbonyl hydroxamates are another family of molecules that are BLRE inhibitors that function by acylation of the enzyme. They are interesting molecules because their structure allows for multiple methods of inhibition. As shown in figure \textbf{1.12}, O-aryloxycarbonyl hydroxamates are prone to hydrolysis through a Lössen rearrangement in the presence of a base\textsuperscript{15}.
Figure 1.12: A Lössen rearrangement is shown, resulting in the formation of an isocyanate.

The isocyanate produced from this rearrangement is very reactive, and could react with the enzyme to form an inert acyl-enzyme adduct\(^1^6\). Additionally, the intact O-aryloxycarbonyl hydroxamate itself can react with a nucleophile in two different ways.

Figure 1.13: Two possible mechanisms of interaction between a nucleophilic enzyme and a carbonate are shown.

The multiple leaving groups present within O-aryloxycarbonyl hydroxamates make them intriguing molecules to use as inhibitors, as acyl enzyme complexes could be formed in a number of different ways\(^1^6\). A variety of O-aryloxycarbonyl hydroxamate derivatives have been tested as inhibitors of BLREs, and it has been shown that they
inhibit the enzymes through the creation of a crosslinked acyl enzyme, one potential mechanism of which is shown in figure 1.14.\textsuperscript{17}

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{figure14.png}
\caption{Potential mechanism of O-aryloxycarbonyl hydroxamate crosslinkage of a BLRE active site.\textsuperscript{17}}
\end{figure}

The mechanism shown above involves crosslinkage of the BLRE active site due to the presence of multiple leaving groups within the O-aryloxycarbonyl hydroxamate inhibitor.

The high level of effectiveness of O-aryloxycarbonyl hydroxamates as inhibitors for BLREs as well as the similarity in proposed mechanisms for BLREs and AS enzymes has led to the examination of the reactivity of AS enzymes with O-aryloxycarbonyl hydroxamates and related molecules.

### 1.7 Inhibitor Design

The following O-aryloxycarbonyl hydroxamate was tested against MAH, and surprisingly it was found to be a substrate of the enzyme.\textsuperscript{18}

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure15.png}
\caption{Compound 1, an example of an O-aryloxycarbonyl hydroxamate that has been shown to interact with MAH.}
\end{figure}
The fact that this molecule can be hydrolyzed by MAH is in contrast with the previously reported literature that indicates that a leaving group larger than methylamine will prevent any interaction with MAH\(^7\). Due to the success of this O-aryloxycarbonyl hydroxamate as a substrate of MAH, the following molecules were synthesized to be tested with MAH.

\[ \text{Figure 1.16: Target molecules synthesized to test with MAH are shown.} \]

These molecules were designed to test both the specificity of MAH as well as the ability of O-aryloxycarbonyl hydroxamates to inhibit MAH, and by extension, AS enzymes. The size of the leaving group in particular is of interest. It would be expected from the literature that compound 2 would be able to interact with the MAH active site better than compound 3, which would be able to interact with the MAH active site better than compound 4, which may possibly not fit within the active site. How MAH interacts with the O-aryloxycarbonyl hydroxamates should help to clarify the active site structure of MAH, and the relationship between AS enzymes and BLREs. The molecules could serve to crosslink the active site, rendering the resulting enzyme inert, serve as substrates of the enzyme, or not react with it at all. Each of these interactions would indicate distinct and interesting qualities regarding the relationship between BLREs and AS enzymes.
Mcleish\textsuperscript{19} showed that 4-nitrophenyl acetate (NPA), with a large para-nitrophenol leaving group, is a MAH substrate. Therefore compound 5 was designed and synthesized.

![Figure 1.17: Molecules to be tested with MAH are shown: 5, methyl p-nitro-phenyl carbonate; 6, 4-nitroacetanilide; 7, Bis (4-nitrophenyl) carbonate.](image)

Compounds 6 and 7 were also examined with regards to their activity with MAH to explore how well molecules with large leaving groups bind to the enzyme. Compound 6 is the amide analog of NPA, while compounds 5 and 7 are analogs of NPA, but, like compounds 2, 3, and 4 contain two leaving groups. The general reactivity of the enzyme with these molecules will help to elucidate the extended specificity and general reactivity of MAH.

### 2. Materials and Methods

#### 2.1 Materials:

Hydroxylamine hydrochloride, benzoic anhydride, benzyl isocyanate, N-succinimidyl N-methylcarbamate, 4-nitrophenyl chloroformate, 4-nitroacetanilide and benzyl chloroformate were purchased from Acros Organics. NADPH, α-ketoglutarate, L-glutamic dehydrogenase, potassium cyanate, Sephadex G-10 beads, FT-IR grade potassium bromide, 4-nitrophenyl acetate, methylamine hydrochloride and potassium
phosphate (monobasic) were purchased from the Sigma-Aldrich Company. Extraction thimbles were purchased from Whatman plc. Analytical and preparative thin layer chromatography (silica gel and alumina gel) were purchased from Analtech Incorporated. Silica gel for columns was purchased from Silicycle Incorporated. Triethylamine was purchased from Fluka Chemical Company. Bis-4-nitrophenyl carbonate was purchased from the Alfa Aesar Company. Magnesium sulfate, sodium bicarbonate, methanol, DMSO and benzene were purchased from the Spectrum Chemical Company. Deuterated DMSO, CD$_3$CN, CDCl$_3$ and D$_2$O were purchased from Cambridge Isotope Laboratories. Potassium carbonate was purchased from the J. T. Baker Chemical Company. Acetone, cyclohexane, hexanes, petroleum ether, diethyl ether, dichloromethane, and ethyl acetate were purchased from the Pharmco-AAPER Company. 1,2-Dichloroethane was purchased from TCI America.

**2.2 Instrumentation:**

**Infrared:** A Perkin Elmer Spectrum BXFT-IR spectrophotometer was used.  
**$^1$H-NMR:** A Varian-300 MHz Nuclear Magnetic Resonance Spectrometer was used.  
**pH:** A Radiometer PHM 220 Lab pH Meter was used.  
**UV-Vis Absorption:** A Hitachi U-2000 spectrophotometer for column chromatography, and an Agilent 8453 spectrophotometer and a Brinkman MGW Lauda RM6 water bath were used to take kinetic measurements.  
**Melting Point:** A Laboratory Devices Mel-Temp Instrument was used.

**2.3 Analytical Procedures:**

**Infrared:** Spectra were taken as KBr pellets.
$^{1}$H-NMR: Deuterated DMSO, CD$_3$CN, CDCl$_3$ or D$_2$O were used as solvents for spectra. Coupling constants in Hz, $J_{ab}$, are reported where determinable.

UV/Vis absorbance spectroscopy: All spectra were taken in 0.1 M K phosphate, 1 mM EDTA buffer, pH 7.8. Cells were equilibrated at 25 °C for each test. 0.5 ml 1 cm path length quartz cuvettes were used. Volumes of solutions were pipetted using Rainin Pipet-Lite pipettes.

2.4 Syntheses and Product Characterization:

2.4.1 O-Carbamoyl Hydroxylamine (NH$_2$OCONH$_2$)

The procedure to obtain O-carbamoyl hydroxylamine was obtained from Zinner$^{20}$. Hydroxylamine hydrochloride (14.10 g, 202.9 mmol) and potassium cyanate (16.23 g, 200.1 mmol) were crushed in a mortar, then mixed with 250 ml methanol in a 500 ml round bottom flask at room temperature for 24 hours. The solvent was removed via rotary evaporation, and the remaining solid was placed in an extraction thimble and placed in a Soxhlet apparatus. An extraction was performed in the Soxhlet apparatus with boiling diethyl ether and a 500 ml round bottom flask as the receiving vessel. The extraction was performed for 8 hours, at the end of which the solvent was removed via rotary evaporation and a fine white solid remained (0.1 g, 1.3 mmol, 0.6% yield).

Characterization of compound O-carbamoyl hydroxylamine:

$^{1}$H-NMR (DMSO): $\delta J_{ab}$: 10.16 (s, 1H), 6.17 (s, 1H) Melting Point 60-68 °C. Lit. 68-70 °C$^{21}$
2.4.2 O-Carbamoyl-N-Benzoyl Hydroxylamine (Compound 2)

The procedure to obtain O-carbamoyl-N-benzoyl hydroxylamine (compound 2) was obtained from Exner\textsuperscript{21}. O-Carbamoyl hydroxylamine (0.1 g, 1.3 mmol) was dissolved in 4 ml dry pyridine in a 10 ml round bottom flask under an N\textsubscript{2} atmosphere and heated to 40 °C. Benzoic anhydride (0.301 g, 1.3 mmol), dissolved in 1 ml dry pyridine, was added to the reaction flask. The reaction was stirred for 10 minutes at 45 °C. The solvent was removed via rotary evaporation. The remaining solid was washed twice with benzene and collected from the benzene suspension by vacuum filtration. The off white solid (0.106 g, 0.59 mmol, 45% yield) obtained was dried overnight on a vacuum pump. \textsuperscript{1}H NMR spectroscopy showed that the remaining solid was a mixture of compound 2, benzoic acid, and pyridine.

Characterization of crude compound 2

Pyridine: \textsuperscript{1}H-NMR (D\textsubscript{2}O): $\delta$ $J_{ab}$ aromatic: 8.59 (d, 2H, 6Hz), 8.38 (t, 1H), 7.84 (t,2H)

Benzoic Acid: \textsuperscript{1}H-NMR (D\textsubscript{2}O): $\delta$ $J_{ab}$ aromatic: 7.94 (d, 2H, 7.5Hz), 7.59 (t, 1H, 6Hz), 7.42 (t, 2H, 9Hz)

Compound 2: \textsuperscript{1}H-NMR (D\textsubscript{2}O): $\delta$ $J_{ab}$ aromatic: 7.76 (d, 2H, 12Hz), 7.42 (t, 1H, 9Hz), 7.33 (t, 2H 7.5Hz)

The required product was isolated by size exclusion chromatography on Sephadex G-10 beads. Fractions were pooled according to absorption spectra, and further characterized via \textsuperscript{1}H NMR experiments. Compound 2 was isolated and existed as a white solid (0.003 g, 0.017 mmol, 1.3% yield).

Characterization of compound 2:

Infrared: 3436 cm\textsuperscript{-1} (N – H); 1700 cm\textsuperscript{-1} (C=O); 1655 cm\textsuperscript{-1} (C=O); 1600 cm\textsuperscript{-1} (C=C aromatic); 1384 cm\textsuperscript{-1} (C – H); 1265 cm\textsuperscript{-1} (C – O). \textsuperscript{1}H-NMR (D\textsubscript{2}O): $\delta$ $J_{ab}$: 7.73 (d, 2H, 9Hz), 7.38 (t, 1H), 7.33 (t, 2H). ESMS+ (H\textsubscript{2}O/CH\textsubscript{3}OH, m/z) 180.87 (M+H)
2.4.3 Benzyl N-Hydroxycarbamate

Hydroxylamine hydrochloride (15.90 g, 228.8 mmol) and potassium carbonate (31.42 g, 227.3 mmol) were added to a mixture of 250 ml diethyl ether and 125 ml water in a 1 L round bottom flask. The mixture was stirred in an ice water bath. After one hour, benzyl chloroformate (21.5 ml, 150.6 mmol) was added to the flask, and the mixture was stirred overnight. The organic layer was separated from the aqueous layer, and the aqueous layer was washed with diethyl ether. The organic layers were combined and dried with sodium sulfate. The solvent was removed from the organic layer via rotary evaporation, and the residue was dried overnight on a vacuum pump. A white solid crystallized overnight (23.13 g, 126.4 mmol, 83.9% yield). This crude product was recrystallized from a 1:1 toluene : cyclohexane solution. White crystals formed, the solution was filtered, and the crystals were placed on a vacuum pump overnight to dry, resulting in pure benzyl N-hydroxycarbamate (18.66 g, 111.6 mmol, 74.1% yield).

Characterization of Benzyl N-hydroxycarbamate:
\[ \delta^1H-NMR (CD_3CN): \delta J_{ab}: 9.70 (s, 1H), 8.73 (s, 1H), 7.34 (m, 5H), 5.04 (s, 2H) \]

2.4.4 O-(N-methylcarbamoyl)-N-Benzylxoxycarbonyl Hydroxylamine (Compound 3)

Benzyl N-hydroxycarbamate (0.753 g, 4.5 mmol) and N-succinimidyl N-methylcarbamate (0.774 g, 4.5 mmol) were mixed with 50 ml dichloroethane in a 100 ml flask. Triethylamine (0.06 ml, 0.44 mmol) was added to the flask dropwise, and the mixture was stirred and brought to reflux for 14 hours. The solvent was removed via rotary evaporation, and the remaining liquid was placed on a vacuum pump.
overnight to remove excess solvent. The resulting product (0.965 g, 96.5% yield) existed as a yellow liquid, and was determined to be a mixture of starting material and product via $^1$H NMR spectroscopy. Attempts to separate the mixture via silica gel column chromatography failed as separation was not sufficient to separate the mixture on a column. 0.100 g mixture was loaded onto an alumina gel preparative TLC plate and a solvent system of 1:1 hexanes : ethyl acetate with 5% methanol was used to separate the mixture. The spot containing compound 3 ($R_f = 0.43$) was removed from the plate, and the silica gel was mixed with methanol, which was then filtered and evaporated to leave a white solid (0.015 g, 0.067 mmol, 15% yield)

Characterization of compound 3:

IR: 3337 cm$^{-1}$ (N – H); 1735 cm$^{-1}$ (C=O); 1500? cm$^{-1}$ (C=C aromatic); 1265 cm$^{-1}$ (C – O); 1237 cm$^{-1}$ (C – O) $^1$H-NMR (CD$_3$CN) $\delta$ $J_{(ab)}$: 8.70 (s, 1H), 7.33 (m, 5H), 5.95 (s, 1H), 5.18 (s, 2H), 2.74 (d, 3H, 6Hz). MP: 60-64 °C.

**2.4.5 O-(N-benzylcarbamoyl)-N-Benzyloxycarbonyl Hydroxylamine**

 (*Compound 4*)

Benzyl N-hydroxycarbamate (0.641 g, 3.8 mmol) was added to a 25 ml round bottom flask and dissolved in 4 ml acetone. The flask was flushed with N$_2$ gas. Benzyl isocyanate (0.47 ml, 3.8 mmol) was added to 2 ml acetone and added dropwise to the reaction flask. The solution was stirred at room temperature for 45 minutes, after which the solvent was removed via rotary evaporation. A clear liquid remained, and the flask was placed on a vacuum pump overnight to remove remaining solvent; after drying, white crystals remained (1.175 g, 3.9 mmol, 103% yield). The crude product was recrystallized from a 1:1 benzene : cyclohexane solution and placed in the cold room (4°C). White solid crystals formed overnight
NMR spectroscopy revealed the recrystallized product to be impure, so separation was attempted via silica gel chromatography. Analytical thin layer chromatography was used to determine a suitable solvent system for separation of the mixture, and a solvent system of 3:1 hexanes : ethyl acetate was chosen. Separation via column chromatography was determined to be ineffective, and preparative thin layer chromatography was used to separate the mixture with a solvent system of 7:3 hexanes : ethyl acetate. 6 preparative plates were used, with 0.100 g of the crude mixture loaded onto each plate (0.600 g total crude mixture used). The appropriate spot ($R_f = 0.55$) was scraped from the preparative plate, and the silica gel was stirred in methanol, then filtered. The methanol was removed from solution via rotary evaporation, and a white solid remained (0.028 g, 0.093 mmol, 4.7% yield from 6 plates).

Characterization of compound 4:

Infrared: 3335 cm$^{-1}$ (N – H); 3300 cm$^{-1}$ (N – H); 1740 cm$^{-1}$ (C=O); 1700 cm$^{-1}$ (C=O); 1540 cm$^{-1}$ (C=C aromatic); 1384 cm$^{-1}$ (C – H); 1250 cm$^{-1}$ (C – O); 1139 cm$^{-1}$ (C – O).

$^1$H-NMR (CD$_3$CN) $\delta$: 10.86 (s, 1H), 8.29 (s, 1H), 7.36 (m, 10H), 5.12 (s, 2H), 4.23 (d, 2H, 6Hz). ESMS$^+$ (H$_2$O/CH$_3$OH, m/z) 323.62 (M + Na). MP: 78-82 °C.

2.4.6 Methyl p-Nitrophenyl Carbonate (Compound 5)

The procedure to obtain methyl p-nitrophenyl carbonate (compound 5) was obtained from Sankaria et al.$^{22}$ Methanol (201 µl, 5 mmol) and triethylamine (692 µl, 5 mmol) were added to 10 ml of anhydrous acetonitrile in a 25 ml flask, and the solution was kept under N$_2$ gas. 4-Nitrophenyl chloroformate (1.005 g, 5 mmol) was added to the mixture, which was then stirred for one hour. Ethyl acetate (15 ml) was added to the mixture, and it was washed with water (3 x 15 ml washes). The organic layer was dried over MgSO$_4$ and then filtered. The solvent was removed via rotary
evaporation, leaving a slightly yellow solid (0.586 g, 3.0 mmol, 59.5% yield). This crude product was recrystallized from a 7:3 ethyl acetate : petroleum ether solution. The resulting product was a white solid (0.203 g, 1.0 mmol, 20.1% yield).

Compound 5: $^1$H-NMR (CDCl$_3$) $\delta$ J$_{ab}$: 8.29 (d, 2H, 6Hz), 7.38 (d, 2H, 6Hz), 3.95 (s, 3H). MP: 110-112 °C Lit: 112-116 °C$^{23}$

2.5 Kinetics of Enzyme Catalysis and Inhibition

All kinetics experiments were carried out in 0.1 M potassium phosphate, 1 mM EDTA buffer (pH 7.8) unless otherwise noted.

The extinction coefficient for 4-nitrophenol was determined by placing a 0.05 mM solution of NPA in a cuvette with buffer, and allowing the cuvette to incubate at 25 °C overnight (14 hours, 50400 seconds). The initial absorbance at 400 nm and the final absorbance at 400 nm were measured, and the difference in absorbances was used to determine the extinction coefficient. To determine the extinction coefficient change on hydrolysis for compound 4 a 0.05 mM solution of compound 4 was placed in a cuvette and allowed to incubate at 25 °C overnight (14 hours, 50400 seconds). The initial absorbance at 260 nm and the final absorbance at 260 nm were measured, and the difference in absorbances was used to determine the change in extinction coefficient.

To determine whether compounds 2, 3, 4, and 5 inhibited MAH, solutions were made containing 15 µl of 7.03 µM MAH and 0.75 µl of 10 mM of the compound in DMSO, resulting in 0.5 mM solutions of the compound to be tested for inhibition and 6.70 µM MAH. These solutions were then incubated at 25 °C. A 3 µl aliquot of each incubation mixture was added to a cuvette, along with 7.5 µl 20 mM
4-nitrophenyl acetate, 7.5 µl DMSO, and 282 µl buffer, resulting in a solution with 0.5 mM NPA, 67 nM MAH, and 5% DMSO. The rate of hydrolysis of NPA was monitored at 400 nm for 600 seconds and compared to the rate of hydrolysis without potential inhibitors added.

To attempt measurement of rates of hydrolysis of compounds 2, 3, 4, 5, and 7, 1.5 µl of a 10 mM stock solution and 13.5 µl DMSO were added to 285 µl buffer, and the reactions were monitored for 14 hours (50400 seconds). The hydrolysis of compounds 2 and 3 proved to be impossible to monitor via direct UV-Vis spectrophotometry, as no reproducible change was observed in the UV-Vis spectrum, so alternative methods were needed to monitor hydrolysis reactions.

In a further attempt to measure the rate of hydrolysis for compound 2, an enzyme coupled assay for ammonia was used. Following the procedure described by Gopalakrishna et al.7 10 µl 4 mM NADPH, 5 µl 16 mM α-ketoglutarate, and 2 µl of 924 units/ml L-glutamic dehydrogenase were added to 88 µl buffer in a 100 µl cuvette. Compound 2 (ca 50 µg) was added to the cuvette, and the reaction was monitored at 340 nm to monitor the conversion of NADPH to NADP. To determine if compound 2 is a substrate of MAH, this procedure was repeated with 1 µl of 140 µM MAH added to the cuvette, and the reaction was again monitored at 340 nm.

To examine the rate of hydrolysis of compound 3, 1 mg of the compound was added to 50 µl CD$_3$CN and 600 µl of 20 mM sodium bicarbonate in D$_2$O, and this solution was monitored via $^1$H NMR. Two peaks were monitored, a peak at 2.93 ppm corresponding to unhydrolyzed compound 3 (CH$_3$), and a peak at 3.14 ppm
corresponding to hydrolyzed compound 3 (CH₃). This procedure was repeated with the addition of 1 µl of 140 µM MAH to examine whether compound 3 is a substrate of MAH.

To test whether compound 4 is a substrate of MAH, 1.5 µl 10 mM stock solution of compound 4, 13.5 µl DMSO, and 3 µl 7.03 µM MAH were added to 282 µl of buffer, and the solution was monitored at 230 nm for 200 seconds.

To determine whether compound 5 is a substrate of MAH, 0.05 mM, 0.1 mM, 0.2 mM, 0.4 mM, 0.5 mM, 0.6 mM, 0.8 mM, and 1.0 mM solutions of compound 5 were made from a 20 mM stock solution of compound 5 in a 300 µl cuvette, along with 3 µl 7.03 uM MAH, and the appropriate amount of DMSO and buffer to make a solution containing 5% DMSO. These solutions were then monitored at 400 nm for 600 seconds, and initial reaction rates were recorded.

To determine whether compound 6 is a substrate of MAH, 7.5 µl of 20 mM compound 6, along with 7.5 µl DMSO and 3 µl 7.03 µl MAH were added to 282 µl buffer in a 300 µl cuvette. The solution was observed at 400 nm for 2000 seconds.

To determine whether compound 7 is a substrate of MAH, 1.5 µl of 20 mM compound 7, along with 13.5 µl DMSO and 3 µl 7.03 µM MAH were added to 282 µl buffer in a 300 µl cuvette. The solution was observed at 400 nm for 2000 seconds. Additionally, the background hydrolysis rate of compound 7 was measured by adding 1.5 µl of 20 mM compound 7 and 13.5 µl DMSO to 285 µl of buffer and observing the reaction at 400 nm for 2000 seconds.
3. Results: Syntheses and Characterizations

3.1 O-Carbamoyl-N-Benzoyl Hydroxylamine (Compound 2)

Scheme 3.1: The synthesis of compound 2 from O-carbamoyl hydroxylamine and benzoic anhydride.

The synthesis of O-carbamoyl hydroxylamine proved to be more difficult than expected following the procedure obtained from Zinner. Initially the reaction of hydroxylamine hydrochloride and potassium cyanate was attempted on a 0.1 mol scale. Extraction of the reaction mixture with boiling diethyl ether did not produce measurable quantities of product even when the extraction was performed for up to 12 hours. When the two reactants were mixed in methanol overnight prior to the extraction the yield increased, but it was still quite low. Doubling the amount of reactants in the extraction also increased the yield, but despite the increased scale of the reaction and stirring in methanol the yield remained very low at 0.64%. Fortunately the product of the reaction appeared to be the required compound as determined via NMR experiments as well as melting point tests.

Once an appropriate amount of O-carbamoyl hydroxylamine had been produced it was reacted with benzoyl chloroformate in dry pyridine to produce compound 2. The crude product obtained from this reaction contained pyridine and a small amount of benzene in addition to other impurities despite attempts to dry it for
multiple days, so further purification was necessary. Initial attempts to separate the product from the impurities involved recrystallization in isopropanol. The crude sample in isopropanol needed to be heated to 40 °C to go into solution, but no solid crystallized despite being placed at 4 °C and then in a freezer at -20 °C. Diethyl ether was added dropwise until the solution turned cloudy, and a small film developed on the bottom of the vial. This was separated from the supernatant, and proved to be nothing of use (no aromatic groups present). Solvent was removed from the supernatant via rotary evaporation, producing a white solid, which was recrystallized from acetonitrile. This recrystallization was placed in a freezer (-20 °C) overnight. A white solid developed overnight, and the supernatant was removed. A 1H NMR experiment in CD$_3$CN showed that the solid was still impure, containing a mixture of compound 2, pyridine, and other impurities. The mixture was dissolved in water, and 0.1% HCl was added until the pH was between 2-4. The organic material was extracted with diethyl ether, but neither the residue nor the ether soluble fraction appeared to be pure. Due to decreasing yields from each attempt at separation this sample was quite small, and another repetition of the experiment was performed.

The next attempt to produce compound 2 proved successful and was described in detail in the materials and methods. The crude product 1H NMR spectrum in D$_2$O (Figure 3.1) contained what appeared to be 3 distinct aromatic compounds. Due to the solvent being D$_2$O, peaks corresponding to protons bound to nitrogens were not expected to exist in the spectrum, making characterization of the compounds present difficult. Each of the three compounds appeared to contain one triplet corresponding to two protons, one triplet corresponding to one proton, and one doublet
corresponding to two protons. Integration values for these peaks were difficult to
determine accurately as the background of the spectrum was quite large, so relative
heights of the peaks was used to determine the amount of protons corresponding to
each peak. The molecules were determined to be from pyridine, benzoic acid, and
compound 2. The peaks corresponding to pyridine were determined to be the triplets
at 8.38 and 7.84, as well as the doublet at 8.59 from a spectrum in D2O only
containing pyridine. The peaks for benzoic acid were determined to be triplets at 7.59
and 7.42, as well as a doublet at 7.94 as determined by a spectrum of just benzoic acid
in D2O. From the assignment of the peaks corresponding to pyridine and benzoic
acid it was determined that the peaks corresponding to compound 2 were triplets at
7.42 and 7.33, as well as a doublet at 7.75. It was determined that the triplet at 7.42
overlapped with a triplet for benzoic acid, as compound 2 should have a triplet in
between the two previously identified peaks corresponding to compound 2.
Figure 3.1: $^1$H NMR of crude compound 2. Solvent is D$_2$O.

Separation of the compounds was successful by Sephadex G-10. Fractions of approximately 0.5 ml were collected and pooled based on absorption spectra

Figure 3.2: UV-Vis spectrum of compound 2 from aqueous column.
The purity of the fractions could be determined from $^1$H NMR spectra, via comparison with the crude spectrum that had been previously assigned. Fractions 65-80 contained pure compound 2 as determined by NMR. The spectrum for these fractions contained a clear doublet at 7.72, which is extremely close to the peak from the crude sample, and very different from the corresponding doublet peaks for benzoic acid and pyridine. Additionally there is a multiplet at 7.35 which appears to contain what could be two triplets that have overlapped. Based on the previously assigned spectra this peak is clearly not benzoic acid or pyridine, so it was expected to be compound 2.

Figure 3.3: $^1$HNMR of pure compound 2. Solvent is D$_2$O

An IR spectrum supported the presence of nitrogen within the sample, as a large broad peak exists at 3436 cm$^{-1}$. Additionally, there are at least two C=O peaks
within the sample. In addition to NMR and IR, ESMS+ was performed on the sample, and a peak at 180.9 M/Z was present in the sample, confirming the existence of compound 2, as the molecular weight of the compound is 180.16 g/mol.

Figure 3.4: IR of compound 2.
Unfortunately the difficulty of producing O-carbamoyl hydroxylamine in a large scale meant that the reaction to obtain compound 2 could only be performed on a small scale (1.3 mmol), so the combination of a low yield step and thus a small scale subsequent reaction led to a very small amount of product (3 mg, 1.3% yield).

3.2 Benzyl N-Hydroxycarbamate

Scheme 3.2: The synthesis of benzyl N-hydroxycarbamate from hydroxylamine hydrochloride, potassium carbonate and benzyl chloroformate.
The synthesis of benzyl N-hydroxycarbamate (scheme 3.2) was straightforward and no major issues were encountered. The crude product, a white solid, crystallized overnight and was determined to contain starting material through $^1$H NMR spectroscopy. This crude product was recrystallized from a 1:1 toluene : cyclohexane solution. White crystals formed resulting in pure benzyl N-hydroxycarbamate as determined by $^1$H NMR spectroscopy (figure 3.5).

Figure 3.6: The $^1$H NMR spectrum of benzyl N-hydroxycarbamate. Solvent is DMSO.

3.3 O-(N-methylcarbamoyl)-N-Benzyloxycarbonyl Hydroxylamine (Compound 3)

Scheme 3.3: The synthesis of compound 3 from benzyl N-hydroxycarbamate and N-succinimidyl N-methylcarbamate.
This synthesis is adapted from Pelto and Pratt\textsuperscript{23} with the inclusion of N-succinimidyl N-methylcarbamate instead of methyl isocyanate because methyl isocyanate is highly toxic. The reaction conditions needed to be modified a few times before a process that produced the desired product in a reasonable time frame was settled on. Initially the reaction was performed in dichloromethane at room temperature, and after one hour no reaction was observed, as shown by an NMR spectrum. The next attempt was in 1,2-dichloroethane at reflux (80 °C), and the reaction was allowed to proceed overnight. Once again no reaction was observed via NMR. Next, a catalytic amount of imidazole (10% molar ratio, 0.44 mmol) was added to the reaction, which was performed in dichloromethane at room temperature. The reaction was allowed to proceed overnight, but again no reaction was observed via NMR. A stronger base, triethylamine (10% molar ratio), was used in the next attempt to catalyze the reaction. Again the reaction was carried out in dichloromethane at room temperature, and the reaction was allowed to proceed overnight. NMR experimentation determined that a small amount of product had been formed, so the stronger base had aided in catalyzing the reaction. To increase the speed of the reaction it was performed in 1,2-dichloroethane at reflux, with triethylamine present in a catalytic quantity. The reaction appeared to proceed mostly to completion overnight.

The crude product, however, contained a small quantity of dichloroethane that could not be removed via vacuum pump, along with N-hydroxysuccinimide, as well as trace amounts of the starting product and other impurities. In the crude product NMR shown below (figure 3.9) it is clear that there are two different sets of benzyl
protons at chemical shifts of 5.13 and 5.18, indicating the presence of two distinct benzyl compounds. Additionally there are four peaks in the general range where protons bound to nitrogens could exist at 5.94, 6.72, 7.98, and 8.69. If the sample were pure there should only be two of these peaks. Additionally, the peak at 2.21 most likely belongs to hydroxysuccinimide. Overall, it was very clear that this sample was not pure, and more separation was necessary.

Figure 3.7: The $^1$H NMR for crude compound 3. Solvent is CD$_3$CN.

Analytical TLC plates were used to determine a suitable solvent system for separation, and it was determined that a very polar solvent system of 1:1 hexanes : ethyl acetate with 3% methanol should separate the required product from starting material. However, a silica gel column did not provide good separation, so the fractions that appeared to contain the most product were run down a second column.
After two columns separation was still not complete. The mixture was finally separated on a preparative alumina plate, which was found to separate the starting material and the product much better than silica gel plates did.

The product recovered from the TLC plate was determined to be pure compound 3. A $^1$H NMR spectrum (figure 3.10) produced a spectrum that contained two peaks corresponding to protons bound to nitrogen at 5.95 and 8.70, a three proton doublet corresponding to the methyl protons at 2.74, a two proton singlet corresponding to the benzyl protons at 5.18, and a five proton multiplet corresponding the aromatic protons at 7.37.

**Figure 3.8:** $^1$H NMR for pure compound 3. Solvent is CD$_3$CN.
The IR spectrum contained the expected broad amide N – H peak at 3337 cm\(^{-1}\) as well as a broad C=O peak at 1735 cm\(^{-1}\). In addition to the amide peaks, C – O peaks are present in the spectrum at 1265 cm\(^{-1}\) and 1237 cm\(^{-1}\), indicating the presence of multiple C – O bonds, consistent with the expectation of a pure sample of compound 3.

**Figure 3.9:** The IR spectrum for compound 3.

### 3.4 O-(N-benzylcarbamoyl)-N-Benzyloxycarbonyl Hydroxylamine (Compound 4)

![Scheme 3.4: The synthesis of compound 4 from benzyl N-hydroxycarbamate and benzyl isocyanate.](image)
This synthesis adapted from Pelto and Pratt\textsuperscript{23} proved to be rather simple, and the reaction ran smoothly. The major issue was not the creation of the product, but rather separation of the product from starting material. The first attempts at purification of the crude product were recrystallizations, first in a 1:1 benzene : cyclohexane solution, followed by an attempt at recrystallization in IPA. The recrystallizations did not fully separate the product from the impurities in the sample, and only served to decrease the yield (from 103\% to 84.9\% to 46.9\%). It was determined that recrystallization would not be useful at this stage of the synthesis, and instead silica gel chromatography was utilized.

Analytical TLC plates were first used to determine an appropriate solvent system to use in a silica gel column for separation; a system of 3:1 hexanes : ethyl acetate was found appropriate to separate the mixture. This column did not separate the mixture as no fraction appeared to contain pure product. Column chromatography was determined to be too imprecise for separation, so preparative plate TLC was used to separate the mixture. A solvent system of 7:3 hexanes : ethyl acetate was used, and this was found to produce good separation of the mixture. Two spots were present on the plates, and it was initially unknown which corresponded to the product, and which corresponded to an impurity in the sample; \textsuperscript{1}H NMR spectra showed that the lower of the two spots contained pure compound 4. The product was subsequently recrystallized from 1:1 benzene : cyclohexane and characterized via IR and MS spectra.

The \textsuperscript{1}H NMR spectrum for compound 4 (figure 3.6) contained peaks at 10.86 and 8.29 which were assigned to the two protons bound to nitrogens of the amide
groups. The presence of a singlet at 5.12 and a doublet at 4.23 confirmed the presence of two separate benzyl groups, and the fact that one exists as a doublet and the other as a singlet was expected as one of the benzyl groups should be split by the proton on one of the nitrogens, while the other is not close enough to any other protons to be split. Additionally the presence of a large multiplet for the two aromatic groups confirmed the presence of compound 4 within the sample.

![Figure 3.10: The 1H NMR spectrum for compound 4. Solvent is DMSO.](image)

The IR spectrum contains multiple peaks in the amide N – H stretch at 3335 cm\(^{-1}\) and 3300 cm\(^{-1}\) and C=O stretch regions at 1740 cm\(^{-1}\) and 1700 cm\(^{-1}\), indicating the presence of two amide groups within the compound. Additionally, there are many peaks in the aromatic region at 1540 cm\(^{-1}\), 1474 cm\(^{-1}\), and 1455 cm\(^{-1}\), indicating the presence of multiple aromatic groups. The mass spectrum (ES+) has a large peak at 323.62, which can be attributed to compound 4 plus residual sodium in the
instrument. The peak at 456.42 is residual material still on the instrument. The various forms of spectroscopy indicate the sample is indeed pure compound 4.

**Figure 3.11:** The IR spectrum for compound 4.
Figure 3.12: The mass spectrum for compound 4.

3.5 Synthesis of Methyl p-Nitrophenyl Carbonate (Compound 5)

Scheme 3.5: The synthesis of compound 5 from 4-nitrophenyl chloroformate and methanol.

The procedure for the synthesis of compound 5 was taken from Sankaria et al.\textsuperscript{23} and was fairly straightforward as described in the materials and methods. The crude product had a slight yellow color, a departure from the literature reported pure white color. The pure product was white, as reported in the literature. The observed
melting point (110-112 °C) was slightly below the literature melting point (112-116 °C), but the $^1$H NMR spectrum in CDCl$_3$ confirmed the presence of sufficiently pure product. The peaks present in the sample were doublets at 8.29 and 7.38, as well as a singlet at 3.95 which match with expected results from the literature.

![Figure 3.13: The $^1$H NMR spectrum for compound 4. Solvent is CDCl$_3$.](image)

### 4. Results: Kinetics Experiments
4.1 Extinction Coefficients

The extinction coefficients for 4-nitrophenol and compound 4 were calculated via the use of Beer’s law, $A = \varepsilon b C$. $A$ represents absorbance, $\varepsilon$ represents molar absorptivity, $b$ represents path length, and $C$ represents concentration. For all calculations the path length was a constant 1 cm. To find extinction coefficients the change in absorbance for a known concentration of sample was divided by the concentration of the sample. For 4-nitrophenol a 0.05 mM solution of NPA was used, and a change in absorbance of 0.755 was measured at 400 nm; using the aforementioned relationship between absorbance, concentration, and extinction coefficient the extinction coefficient was found to be $15100 \text{ M}^{-1}\text{cm}^{-1}$. For compound 4 a change in absorbance of 0.0151 was observed at 260 nm in a 0.05 mM sample, resulting in an extinction coefficient change of $302 \text{ M}^{-1}\text{cm}^{-1}$

4.2 Inhibition Tests

Compounds 2, 3, 4, and 5 were all tested as covalent inhibitors of MAH, but no inhibition was observed. Full MAH activity with NPA was observed when 0.5 mM of all four compounds were incubated with MAH for 0, 1, 2, 3, and 24 hours.
4.3 Substrate Tests

Compound 4 was found to not be a substrate of MAH because there was no significant difference between the rate constant for the hydrolysis of compound 4 when MAH is present or is absent (Table 4.1).

<table>
<thead>
<tr>
<th></th>
<th>Hydrolysis Rate Constant (k) (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncatalyzed Reaction</td>
<td>6.38 x 10⁻³ ± 4.33 x 10⁻⁴</td>
</tr>
<tr>
<td>Reaction in the presence of MAH</td>
<td>6.41 x 10⁻³ ± 2.62 x 10⁻⁴</td>
</tr>
</tbody>
</table>

Compound 3 was determined to be a substrate of MAH due to the difference in rate constants for the catalyzed hydrolysis by MAH and the uncatalyzed hydrolysis in buffer, determined from $^1$H NMR experimentation.

The catalyzed reaction would have a general scheme as follows:

$$E + S \xrightarrow{k_{enz}} E + P$$

While the uncatalyzed reaction would be simply:

$$S \xrightarrow{k_o} P$$

The rate constant determined by observation of the catalyzed reaction would be as follows:

$$k_{obs} = k_{enz} + k_o$$

Using the above relationship, the value of $k_{enz}$ can be found (Table 4.2).
### Table 4.2: Rate constants for catalyzed and spontaneous compound 3 hydrolysis

<table>
<thead>
<tr>
<th>Reaction Type</th>
<th>Hydrolysis Rate Constant (k) (s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncatalyzed Reaction (k(_o))</td>
<td>(2.38 \times 10^{-4} \pm 2.27 \times 10^{-4})</td>
</tr>
<tr>
<td>Catalyzed Reaction (k(_{obs}))</td>
<td>(3.35 \times 10^{-4} \pm 5.12 \times 10^{-5})</td>
</tr>
<tr>
<td>Enzyme Kinetic Constant (k(_{enz}))</td>
<td>(9.7 \times 10^{-5} \pm 4.11 \times 10^{-4})</td>
</tr>
</tbody>
</table>

\(k_{cat}/K_m\) can then be determined from the following relationship:

\[
k_{enz} = (k_{cat}/K_m)e_0
\]

The initial enzyme concentration \((e_0)\) for compound 3 was 0.216 µM; using this value, along with the determined \(k_{enz}\), \(k_{cat}/K_m\) was found to be \(4.49 \times 10^2\) M\(^{-1}\) s\(^{-1}\).

Compound 5 was also found to be a substrate of MAH, and measurements of the initial rate of catalyzed hydrolysis were carried out at a variety of concentrations of compound 5. The results of these tests were then used to create a Michaelis-Menten plot of the data (Figure 4.2), from which the steady state kinetic parameters of the interaction between compound 5 and MAH were determined (Table 4.3).
Figure 4.2: Michaelis-Menten plot for compound 5.

<table>
<thead>
<tr>
<th>Table 4.3: Kinetic Parameters for Compound 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{\text{max}}$</td>
</tr>
<tr>
<td>$5.68 \times 10^{-4} \pm 6.25 \times 10^{-5}$ As$^{-1}$</td>
</tr>
</tbody>
</table>

$k_{\text{cat}}$ was determined by dividing $V_{\text{max}}$ by the extinction coefficient for 4-nitrophenol (15100 M$^{-1}$cm$^{-1}$) and the concentration of enzyme (0.073 µM).

Compound 6 was found to not be a substrate of MAH, as no absorption change reflecting hydrolysis was noted when compound 6 was incubated with MAH.

Compound 7 was found to be either a very poor substrate of MAH, or not a substrate at all. There was no noticeable increase in the rate of hydrolysis of compound 7 when MAH was present. However, due to the very fast rate of
hydrolysis of compound 7 when it is alone in buffer it is possible that it could still be a poor substrate without a noticeable change in the observed reaction rate. The upper bound of the kinetic parameter $k_{\text{cat}}/K_m$ was thus estimated from 20% of the rate for the observed reaction, as shown below.

$$k_{\text{cat}}/K_m \leq (0.2) \frac{v_o}{(e_o s_o)}$$

The $v_o$ in this equation is the initial rate for the hydrolysis of compound 7, measured to be $7 \times 10^{-5}$ As$^{-1}$, which converts to $2.27 \times 10^{-9}$ Ms$^{-1}$. Using the equation above the maximum value of $k_{\text{cat}}/K_m$ was found to be $65$ M$^{-1}$s$^{-1}$.

4.4 Problems with Compound 2

No spontaneous hydrolysis reaction could be observed spectrophotometrically for compound 2. Additionally, in the coupled enzyme assay performed on compound 2, no appearance of ammonia that should accompany hydrolysis was observed. No hydrolysis was observed of compound 2 at all, which was not expected, as compounds 3 and 4 spontaneously hydrolyze in buffer at measurable rates, and it would be expected due to those results that compound 2 should hydrolyze as well. No reaction was observed in the presence of MAH either. The fact that no spontaneous or catalyzed hydrolysis of compound 2 was observed suggests that compound 2 may have decomposed in between the point at which it was characterized via NMR, IR, and MS spectra and the kinetic experiments were performed on it.
5. Discussion

5.1 Syntheses and Characterization

The syntheses and characterization of compounds 3, 4, and 5 were fairly straightforward, and the characterization by NMR, IR, and MS all supplied convincing evidence that the syntheses were carried out successfully and the compounds isolated were indeed sufficiently pure.

The synthesis and characterization of compound 2 is slightly less conclusive. The spectroscopic data all indicate that compound 2 was isolated, and was pure at the time of isolation. The MS data indicates that a pure compound with a molecular weight of 180.9 g/mol, which is the expected molecular weight of compound 2, was isolated. Additionally, the NMR spectrum indicated that there was just one molecule present in the sample containing a benzyl group, and comparisons to other spectra indicated fairly clearly that the molecule present was not any of the supposed impurities in the crude sample. The IR data as well contains all of the expected peaks. However, by the time that kinetic experiments were done on compound 2 after a period of 2 months stored at -20 °C it seems as though the sample had decomposed. This was suspected because not even a background rate of hydrolysis for compound 2 could be determined. Additionally, based on the success of compound 3 as a substrate for MAH, and the published literature\(^7\) that suggests that primary amides will react more readily with MAH than will secondary amides, it seems as though compound 2 should definitely interact with MAH, if not as an inhibitor than at least as a substrate. Thus, it is probable that compound 2 is not very stable, and
decomposes readily. The likely products of decomposition of compound 2 are shown below.

Scheme 5.1: The decomposition of compound 2.

5.2 Kinetics

The results from the kinetics experiments performed on compounds 3 and 4 were consistent with results produced by Gopalakrishna et al. According to the literature, a leaving group larger than methylamine will prevent a molecule from binding to MAH. This explains why compound 3 is a substrate, while compound 4 is not; the only difference between the two molecules is the presence of a benzyl group on compound 4 as compared to a methyl group on compound 3.

When $k_{cat}/K_m$ for compound 3 is compared to those for a variety of other amides (Table 5.1) that are hydrolyzed by MAH, it is clear that compound 3 is not a very good substrate of MAH. However, the data also suggests that compound 3 suffers from the presence of a larger leaving group than most other substrates of MAH. When compared to another substituted amide, such as N-methyl phenylacetamide, the $k_{cat}/K_m$ values for the two molecules are very similar despite there being a large difference in the size of the two molecules. This suggests that while the MAH active site has a very high specificity with regards to the size of the
leaving group and substitution of the amide nitrogen, the active site still allows for the binding of a wide range of molecule sizes.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>$k_{cat}/K_m (\text{M}^{-1}\text{s}^{-1})$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Phenylacetamide</td>
<td>$3.77 \times 10^6$</td>
</tr>
<tr>
<td>R Mandelamide</td>
<td>$4.75 \times 10^5$</td>
</tr>
<tr>
<td>S Mandelamide</td>
<td>$4.53 \times 10^5$</td>
</tr>
<tr>
<td>Hexanoamide</td>
<td>$1.74 \times 10^3$</td>
</tr>
<tr>
<td>N-Methyl Phenylacetamide</td>
<td>$6.98 \times 10^2$</td>
</tr>
<tr>
<td>Compound 3</td>
<td>$4.49 \times 10^2$</td>
</tr>
</tbody>
</table>

Unfortunately, compound 2 could not be properly tested with MAH, as it most likely decomposed. Had it been tested, it would have been expected to be a better substrate than compound 3, as compound 2 has a smaller leaving group. Once again, comparing compounds 2 and 3 to previously examined substrates of MAH indicates that a methylamine leaving group as compared with an ammonia leaving group can decrease $k_{cat}/K_m$ by up to 4 orders of magnitude (Table 5.1).7

The lack of inhibition by compounds 3 and 4 indicates that they were unable to form stable acyl enzymes with MAH, and that hydrolysis occurs without the presence of a stable covalent intermediate. While O-aryloxycarbonyl hydroxamates have shown the ability to crosslink active site residues in BLREs, it seems as though this does not happen with AS enzymes. Instead, catalysis is most likely completed as
shown below, with release of the substrate occurring when a water molecule attacks the acyl-enzyme:

![Diagram](image)

**Figure 5.1:** Mechanism for AS enzyme breakdown of compound 3. 1 shows the suspected mechanism, which does not cross link the enzyme. 2 shows the mechanism by which the enzyme is crosslinked.

The results from the experiments on compound 5 were interesting. NPA has previously been shown to be a substrate of MAH\(^{10}\), and the leaving group of NPA hydrolysis must be p-nitrophenol. This leads to the question of the conformation that NPA takes within the MAH active site. Generally the leaving group of the substrate fits into what has been come to be recognized as a tight binding pocket in the active site that would not fit a nitrophenol leaving group. The fact that NPA is a substrate of MAH indicates that the leaving group of the molecule may not need to fit into the small portion of the active site, rather that substrates can bind in one of two conformations within the active site.
Figure 5.2: Examples of conformations that substrates take within the MAH active site. **A**: Phenylacetamide, with the ammonia leaving group bound to the small, specific region of the active site; **B**: p-Nitrophenyl acetate, with the p-nitrophenol leaving group bound to the large, non-specific region of the active site.

This interpretation of the NPA results with MAH leads to the question of what orientation compound 5 binds to MAH in, and which of the two possible leaving groups leaves first (Figure 5.3). Additionally, that the $k_{cat}/K_m$ of compound 7, which only possesses a p-nitrophenol leaving group, has a value less than $65 \text{ M}^{-1}\text{s}^{-1}$ means that it is unlikely that a p-nitrophenol leaving group can fit into the MAH active site.

Figure 5.3: The two potential leaving groups of compound 5 are shown.

Compound 5 has a larger substituent on the carbonyl carbon than NPA, which is similar to having a larger substitution on an amido nitrogen. When the $k_{cat}/K_m$
values for the two molecules are compared, it is clear that they are both substrates for MAH, but, NPA is a slightly better substrate than compound 5 (Table 5.2).

<table>
<thead>
<tr>
<th>Table 5.2: Kinetic Parameters for Compound 5 and NPA</th>
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<tbody>
<tr>
<td>kcat (s⁻¹)</td>
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<td>Km (mM)</td>
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<tr>
<td>kcat/Km (M⁻¹s⁻¹)</td>
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The results for compound 6 are also useful in confirming the suggested high specificity of MAH. Compound 6 has an amide substituted with a phenyl ring, which is significantly larger than the expected binding pocket of MAH; the fact that no reaction did occur is another indication that the MAH binding pocket is small and specific. The fact that compound 6 is an isostere of NPA, and would thus be expected to bind to MAH in the same way as NPA indicates that a good leaving group, such as p-nitrophenol, is necessary for hydrolysis to occur if the leaving group does not fit into the amide pocket. p-Nitrophenol is a good leaving group because it can leave without the presence of a proton to catalyze hydrolysis, whereas the aniline leaving group of compound 6 requires a proton to catalyze hydrolysis of the amide bond.

Overall, the results help to confirm the theory that MAH has a small and highly specific active site where catalysis occurs, but the rest of the active site is large and tolerates a wide variety of molecules. The ability of compound 3 to interact with MAH, while compound 4 cannot, confirms that amide leaving groups must be small. That compound 5 can be hydrolyzed by MAH while compound 6 cannot indicates
that large leaving groups are tolerated as long as they are good leaving groups that do not require a proton. Additionally, the fact that compound 3 is a substrate of MAH indicates that MAH can tolerate substrates of varying sizes. Also, that the active site does not crosslink during the breakdown of an O-aryloxycarbonyl hydroxamate, compound 3, like BLREs do indicates that the nucleophile necessary for crosslinkage does not have access to the acyl-enzyme intermediate. This is the case despite the high levels of similarity in position for the catalytic residues in AS enzymes and BLREs.

6. Future Work

The success of compound 3 as a substrate of MAH is encouraging, and indicates that O-aryloxycarbonyl hydroxamates can, and will, interact with MAH. Testing compound 2 on MAH would be a logical next step to take, while care must be taken to ensure that it does not decompose as it has been shown to be reasonably unstable. While no noticeable inhibition was observed when compound 3 was incubated with MAH, further experimentation should be completed with O-aryl hydroxamates to attempt to crosslink the active site in a similar manner as is observed in BLREs. It is clear that MAH goes through an acyl-enzyme intermediate in the breakdown of substrates such as compound 3 and compound 5; unfortunately these intermediates do not appear to crosslink within the enzyme. The lack of crosslinking in the breakdown of compounds 3 and 5 indicates that despite the similarity in active site residues, and possibly mechanism, between BLRE and AS active sites differences still exist. If crosslinking were able to be achieved it would be an indication that the
catalytic residues in the active sites of BLREs and AS enzymes have similar amounts of flexibility. If no crosslinking can be achieved it would indicate that AS enzymes have a more rigid active site structure and catalytic residue conformations.
References

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Appendix

Figure A1: UV-Vis spectrum for compound 2

Figure A2: UV-Vis spectrum for compound 3
Figure A3: UV-Vis spectrum for compound 4

Figure A4: UV-Vis spectrum for compound 5
Figure A5: Hydrolysis of compound 3 with MAH present. Line of best fit according to a pseudo first order equation is shown.

Figure A6: Hydrolysis of compound 3 in buffer. Line of best fit according to a pseudo first order equation is shown.
Figure A7: Hydrolysis of compound 4 with MAH present. Line of best fit according to a pseudo first order equation is shown.

Figure A8: Hydrolysis of compound 4 in buffer. Line of best fit according to a pseudo first order equation is shown.