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α -HYDROXYHIPPURIC ACID DERIVATIVES: *pH*-DEPENDENT AQUEOUS
KINETICS AND BUFFER CATALYSIS.

Kati Feken

88 Pages

December 2013

Carbinolamides have been shown to be involved in a variety of biological systems. Our interest is focused on their role as intermediates and the mechanism of their catalytic breakdown in the synthesis of α -amidated peptide hormones by the enzyme; peptidylglycine α -amidating monooxygenase. Approximately 50% of all known mammalian peptide hormones possess a C-terminal α -amide which is critical for the proper function of the peptide hormone. The studies here were focused on aromatic substituted derivatives of α -hydroxyhippuric acid with the goal of determining the effect of the carboxylic acid group on the reactivity and mechanism of reaction for these compounds in water as a function of *pH*.

The α -hydroxyhippuric acid compounds are known substrate for the enzymatic system itself. Generating a better understanding of how these compounds undergo reaction may allow for a better understanding of the enzyme catalyzed reaction. Practically nothing is known about the reactivity of this class of carbinolamides in aqueous solution. Studying the effects of the added carboxylate and varied substituents on the acid and base dependent reactions and extending these studies to the enzymatic

system may allow us to understand more of the details of the enzyme catalyzed reaction. The studies will provide the foundation to answering these questions and many others.

An initial focal point of this research was the synthesis and purification of a series of aromatic substituted α -hydroxyhippuric acid compounds. Procedures exist for the synthesis of the parent compound (α -hydroxyhippuric acid), however, the addition of substituents caused synthetic and purification complications. Following the synthesis of the target compounds, the study of the solution kinetics as function of pH was initiated. A number of techniques were employed to follow the rates of these reactions; UV-Vis spectrophotometer, stopped-flow spectroscopy, and HPLC.

α -HYDROXYHIPPURIC ACID DERIVATIVES: *pH*-DEPENDENT AQUEOUS
KINETICS AND BUFFER CATALYSIS.

KATI FEKEN

A Thesis Submitted in Partial
Fulfillment of the Requirements
for the Degree of

MASTER OF SCIENCE

Department of Chemistry

ILLINOIS STATE UNIVERSITY

2013

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α -HYDROXYHIPPURIC ACID DERIVATIVES: *pH*-DEPENDENT AQUEOUS
KINETICS AND BUFFER CATALYSIS.

KATI FEKEN

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K. A. F.

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CHAPTER I
AN INTRODUCTION OF THE CARBINOLAMIDE FUNCTIONALITY

Carbinolamides (**1**) are a functionality generated by the reaction of an amide and an aldehyde, followed by a proton shift. While not a particularly well known functionality, reports of these compounds have appeared in the literature as early as the 1870's.¹ Most of the early work concerning carbinolamides focused on highly electrophilic aldehydes with the primary aldehyde of interest being formaldehyde.² Historically, interest in the area has focused on their use for synthetic purposes.² Their primary synthetic role has been to act as a source of electrophilic carbon for aromatic substitution reactions in an acid catalyzed breakdown of the carbinolamide to produce amidinium ion intermediates (Scheme 1; Tscherniac-Einhorn reaction).² Synthetic issues and the lack of convenient routes to form a variety of carbinolamides has led to the Tscherniac-Einhorn reaction receiving little interest. More recently, carbinolamides have

been shown to be important intermediates in a variety of biological venues and critical to the bioactivity of a number of compounds.²⁻¹²

Scheme 1: Tscherniac-Einhorn Reaction

One area where a carbinolamide has been found to have an interesting role is in the commercially available antibiotic bicyclomycin (**2**). Bicyclomycin is not a broad spectrum antibiotic but it has been shown to inhibit the growth of gram-negative bacteria.²⁻¹² This particular antibiotic is thought to have a unique form of reactivity due to the bioactivity of the critical carbinolamide functional group (highlighted in blue, see **2**). Studies investigating bicyclomycin's mode of action have concluded that **2** inhibits rho-dependent termination processes by binding to a cleft in the rho-protein that includes the

site for ATP hydrolysis.¹³⁻²¹ Further evidence suggested that **2** covalently attaches, however, the nature of this covalent modification and mechanism by which it occurs has not been determined.⁷

The outcome described above couples well with potential modes of reactivity that were observed in previous studies that investigated the reactivity of bicyclomycin at a variety of *pH* values in the presence of nucleophiles (Scheme 2). One study, which was performed in 3:1 THF/H₂O in the presence of thiolate nucleophiles, proposed a mode of bioactivity based upon the products of the reaction isolated and identified. Scheme 2 shows the proposed mechanism for the reaction of **2** in the presence of thiolate nucleophiles that was used to explain the outcome of the reaction.⁷ The proposed mechanism has the carbinolamide reversibly opening to unmask an α,β -unsaturated ketone. Once unmasked, the α,β -unsaturated ketone would be susceptible to nucleophilic attack and covalent modification (see Scheme 2). After nucleophilic attack on the double bond and tautomerization of the subsequently generated enol, the carbinolamide reforms to yield the product shown in Scheme 2. Whether the mechanism shown in Scheme 2 represents a true mode for the bioactivity for **2** is unknown but several aspects of the reactivity are unique and interesting.

Scheme 2: Proposed Mechanism of Bicyclomycin with Thiols

Scheme 3: Reaction of Guanine with Glyoxal to Form an Etheno Base Adduct

A forum where carbinolamides are thought to have a negative role is in the interaction of glyoxal with DNA.²²⁻²⁶ The carbinolamide intermediate generated from the reaction of endogenously or exogenously generated dialdehydes and a nucleic acid leading to the carbinolamide intermediate (**3**) shown in Scheme 3. This reaction has been shown to occur between guanine and glyoxal but no information concerning the order in which the carbinolamide and carbinolamine are formed was discussed. After the formation of **3**, a further reaction/series of reactions modifies the “diol” into the etheno bridged species (see **4** in Scheme 3). Such modification of nucleic acids can lead the organism to being susceptible to mutations and replication errors.²⁷⁻³² Shown in Scheme 3 is the transformation of guanine to 1,N²-ethenodeoxyguanine. In forming the etheno

bridge, the added ring structure can interfere with proper base pairing, leading to RNA transcription errors in regions where this modification is present. Without proper transcription of DNA, mutations in the organism result and it has been shown that increased amounts of cyclic etheno and propanobase adducts in individuals have been linked with high risk of certain cancers.^{31,32}

Scheme 4: Carbinolamide Breakdown into Urea and Glyoxylic Acid

Another compound related to carbinolamides is ureidoglycolate (**5**) that is catalytically decomposed in one of the final steps in the catabolic pathway leading from purines to urea (see Scheme 4).²³ Ureidoglycolate lyase (UGL) has been found in many bacteria, yeast and fish but it is not generally found in higher mammals. The enzyme UGL has received relatively little attention but it has been established that Mn(II) restores activity of the enzyme after treatment with EDTA. This result was particularly interesting as Mn(II) was one of the metals that was shown to restore the activity of peptidylamidoglyoxylate lyase (see below). Ureidoglycolate, while not formally a carbinolamide, is structurally very similar to carbinolamides and its reactivity is likely to have many parallels with the reactivity of carbinolamides.

Of particular interest was the discovery that carbinolamides are essential intermediates (**7**) for enzymatic synthesis of peptide hormones. Peptidylglycine α -

amidating monooxygenase (EC 1.14.17.3, PAM) is a bifunctional enzyme that mediates the transformation of the glycine extended peptide precursor (**6**) to the biologically active C-terminal α -amidated peptide hormones (**8**) and glyoxylic acid (**9**) (Scheme 5).^{33,34}

Scheme 5: Mechanistic Pathway of PAM with Glycine Extended Precursors

Approximately half of the known mammalian peptide hormones require a C-terminal α -amide functionality in order to be biologically active which makes this transformation a critical process.³⁵ In some cases, lack of a C-terminal α -amide can have lethal consequences. PAM, a bifunctional enzyme, contains two distinct enzymatic systems which ultimately catalyze the transformation of the glycine-extended peptide precursor. The individual enzymes have also been isolated and are peptidyl α -hydroxylating monooxygenase (EC 1.14.17.4, PHM) and peptidylamidoglyoxylate lyase (EC 4.3.2.5, PAL). This system is the only pathway known to provide this C-terminal α -amide functional group.^{34,39} The first step in the generation of the C-terminal α -amide functional group is the oxidization of the pro-*S* hydrogen of the glycine extended peptide precursor (**6**) to yield the carbinolamide intermediate (**7**), in the PHM active site. The oxidative step, in the PHM active site, requires two copper (II) atoms, molecular oxygen, and ascorbate.⁴¹ Much of the primary mechanistic studies performed on PAM have

focused on the oxidative step and very few have studied PAL which catalyzes the breakdown of the carbinolamide.

The second step in the formation of α -amidated peptide hormones is the breakdown of the carbinolamide intermediate into the peptide hormone and glyoxylic acid. This transformation is mediated by the Zn(II)- dependent PAL enzyme where the carbinolamide is catalytically cleaved to form the bioactive peptide hormone and glyoxylic acid. It is not known if the Zn^{2+} is a structural component in the enzyme or involved in catalysis itself (this will be discussed in greater detail below). As stated above, the majority of the mechanistic enzymatic studies of this transformation have focused on the first reaction involving PHM but little attention has been placed on the mechanism of catalysis involving PAL. This oversight may, in part, be explained by a lack of understanding of the carbinolamide functionality itself.

More recently, an ever increasing number of compounds, having interesting biological function, are being discovered and developed, which contain the carbinolamide functionality. For example, talaroconvolutin, azaspirene, UCS1025A, oteromycin, and epolactaene all contain carbinolamides and have a wide variety of potential uses.⁴² The role that the carbinolamide plays in each of these compounds has not been defined but this growing list of compounds possessing a carbinolamide functionality suggests that a broader understanding of carbinolamide reactivity is a necessity.

Previous Mechanistic Studies:

It is obvious why there would be interest in studying carbinolamides given their positive and negative attributes in biological reactions. As previously mentioned, a gap in the mechanistic knowledge of the PAL catalytic breakdown of carbinolamides to form activated peptide hormones exists. In part, the reason for this gap in the understanding of this catalytic process is due to a lack of understanding involving the mechanisms by which carbinolamides breakdown. The majority of our understanding of the reactivity of carbinolamides was the result of mechanistic studies performed on carbinolamides in an aqueous environment with the goal of probing their potential as pro-drugs.^{38,43-45}

Pro-drugs are substances that are inactive due to a protective group attached to the molecule that will undergo chemical transformation to an activated “parent” compound *in vivo*.^{38, 43-45} Compounds of this type are of interest because they can improve drug efficacy and safety for a couple of reasons. One of the goals is for the pro-drugs to have greater solubility than their activated forms. This greater solubility allows the substance to be absorbed more readily and then, once activated, perform the role of the parent compound but at a greater concentration than could be achieved by the parent compound itself. Alternatively, the transformation of the parent compound into the pro-drug results in a decrease in the rate of their metabolism in the organism, allowing it time to reach its place of bioactivity or to have a more sustained effect within the organism itself. Ultimately, pro-drugs could lead to decreased amount of overdosing or buildup in the biological system.^{38, 44-45}

Several different types of *N*-acyloxyalkylated groups which included a class of

carbinolamides, the *N*-(hydroxyalkyl)benzamide derivatives, were studied as potential pro-drugs. Shown below, in Figure 1, are examples of several types of carbinolamides that were investigated in a series of studies by Bundgaard *et. al.*⁴³⁻⁴⁵ An abbreviated *pH*-rate profile was generated for several of the derivatives shown in Figure 1. While the studies did not cover the entire *pH*-rate profile, the results did show some interesting trends. At low to moderate hydroxide concentrations, there was a hydroxide-dependent region. In the neutral region, there was a *pH*-independent reaction and at high acid concentrations, there was an acid-catalyzed region. These initial mechanistic studies gave rise to the proposed mechanisms of breakdown of carbinolamides in water (see Schemes 6-7).

N-(Hydroxymethyl)benzamide

N-(Hydroxymethyl)thiobenzamide

N-(Hydroxybenzyl)benzamide

N-(Hydroxyethyl)thiobenzamide

N-(Hydroxy-2,2,2-trichloroethyl)benzamide

N-(Hydroxy-2,2,2-trichloroethyl)thiobenzamide

Figure 1: Various *N*-(Hydroxyalkyl)benzamides Used in Bundgaard Study

Scheme 6: Accepted Mechanism for the Hydroxide-Dependent Breakdown of Carbinolamides

$$(k_{\text{obsd}})^{\text{OH}^-} = k'_1[\text{OH}^-] = k_1 K_a [\text{HO}^-] / K_w \quad (1)$$

Scheme 7: Specific-Acid Catalyzed Mechanism

The proposed hydroxide-dependent mechanism of carbinolamide breakdown, as a result of the pro-drug studies, is shown in Scheme 6.⁴³⁻⁴⁵ The initial reaction is a specific-base catalyzed deprotonation of the hydroxyl group, generating the conjugate base (**10**). Then, in the rate limiting step, **10** undergoes an elimination to generate the aldehyde product and an amidate leaving group (**11**). The amidate is then protonated by solvent in a post-rate limiting step (see Scheme 6). The conclusion that the reaction occurred by a specific base catalyzed mechanism was due to curvature in plot of k_{obsd} vs. $[\text{HO}^-]$ at higher [hydroxide] (see Figure 2), and the absence of buffer-catalysis in the hydroxide dependent reaction. Bundgaard *et al.* only reported the apparent second-order rate constants (k'_1) for the hydroxide-dependent reaction from the linear portion of the k_{obsd} vs. $[\text{HO}^-]$ plot.^{38,43-45} The apparent second-order rate constant is a complex value that incorporates both the K_a of the hydroxyl group and the rate for the decomposition of **10** (see eq. 1). In order to truly understand the reactivity of carbinolamides, both k_1 and K_a must be determined so that the effect of structural changes can be detailed (see eq. 2). As a result, further investigation was needed because those variables could not be calculated using the Bundgaard *et al.* studies alone.

$$(k_{\text{obsd}})^{\text{HO}} = k_1 \frac{K_a[\text{OH}^-]}{K_w + K_a[\text{OH}^-]} \quad (2)$$

Studies by Tenn *et al.* probed the full pH -rate profile of N -(hydroxymethyl)benzamide derivatives, thus allowing for the determination of k_1 and K_a (Figure 2 & Equation 2).³⁶ The pH -rate profile shown in Figure 2 exhibits four distinct regions of reaction. There is an acid-catalyzed region, a water-catalyzed region, a hydroxide-dependent region and a hydroxide independent region at higher pH . In the pH range from 0 to 3, there is a first order dependence on the acid concentration present. It is thought that region 3 to 6 is the water-catalyzed region while the pH values from 6.5 to 12 is hydroxide-dependent. For the region with $pH > 12$ there is a hydroxide-independent reaction occurring.

Figure 2: The pH rate profile for the aqueous decomposition of N -(hydroxymethyl)benzamide (▲), 4-chloro- N -(hydroxymethyl)benzamide (●), and 2,4-dichloro- N -(hydroxymethyl)benzamide (■), in H_2O , $I=1.0M$ (KCl), at $25^\circ C$ ³⁶

These studies included a careful investigation of the hydroxide-independent region of the pH rate profiles of the N -(hydroxymethyl)benzamide derivatives. Figure 3, which depicts how k_{obsd} changes with increasing $[\text{HO}^-]$ and it is evident that, at approximately 0.2-0.3M, there is a switch from first order hydroxide-dependence to a zero-order dependence. Further, the data indicate that as electron withdrawing groups are added, the rate at which the compounds react also increases.

Figure 3: Observed rate of breakdown versus hydroxide concentration of N -(hydroxymethyl)benzamide (▼), 4-chloro- N -(hydroxymethyl)benzamide (●), and 2,4-dichloro- N -(hydroxymethyl)benzamide (■) in H_2O , $I=1.0$ M (KCl), at 25°C ³⁶

As previously mentioned, the hydroxide-dependent reaction is a specific base catalyzed reaction. A specific base/acid catalyzed reaction is one in which the rate of reaction is dependent on the concentration of base/acid but proton transfer does not occur

in the rate-limiting step. As shown in Scheme 6, increases in the amounts of hydroxide shifts the equilibrium to the right (forming the conjugate base of the carbinolamide). It is the conjugate base of the carbinolamide that undergoes rate-limiting breakdown generating the aldehyde and amidate. Thus, increases in the amount of hydroxide lead to an increase in the concentration of the species undergoing rate-limiting breakdown. Once the pK_a of the hydroxyl group has been passed, further increases in the concentration of hydroxide result in no change in observed rate of the reaction (hydroxide independent region, see Figures 2 & 3). The data in Figure 3 can be fit to equation 2 to obtain the K_a of the hydroxyl group and the rate-limiting breakdown of the alkoxide of the carbinolamide (k_1). Shown in Figure 4 are results of hydroxide-dependent breakdown of various *N*-(hydroxymethyl)benzamide derivatives with electron withdrawing groups. The results shown in Figure 4 illustrate that the addition of substituents to the aromatic ring of the amide have little to no effect on the pK_a of the hydroxyl group of the carbinolamide.³⁶ Conversely, the substituents have a significant effect on the rate-limiting departure of the amidate (a 5-fold increase over the compounds studied). Departure of the amidate would lead to significant build up of charge on the nitrogen which could be inductively stabilized by the addition of electron-withdrawing groups to the aromatic ring of the carbinolamide derivatives. This explanation has been used to explain the increase in k_1 in the hydroxide-dependent reaction upon the addition of electron-withdrawing groups.

R	p <i>K</i> _a	<i>k</i> ₁ (s ⁻¹)
13.05	0.042
13.04	0.068
13.02	0.070
12.90	0.087
13.20	0.14
13.00	0.13
13.10	0.16
13.04	0.19

Figure 4: p*K*_a's and *k*₁ Values of N-(Hydroxymethyl)benzamide Derivatives³⁶

The acid-catalyzed mechanism of carbinolamide breakdown has not been subject to extensive investigation. The results of the pro-drug studies led to the proposed specific-acid mechanism shown above (Scheme 7).^{38,43-45} This conclusion received support from some an initial report by Tenn *et al.* in which no buffer catalysis was observed for studies involving the *N*-(hydroxymethyl)benzamide derivatives in the

presence of a variety of buffer concentrations from 0.02-0.10M at an ionic strength ($I = 1.0\text{M}$).³⁶ The specific-acid catalyzed reaction was proposed to occur by first the reversible protonation of the carbonyl oxygen (**12**), followed by rate limiting decomposition to form a tautomer of the amide and the protonated aldehyde.

More recently, studies from the Nagorski group have shown that the acid-catalyzed reaction is not a specific-acid reaction as proposed by Bundgaard *et al.*⁴⁵ More complete buffer studies with *N*-(hydroxymethyl)benzamide derivatives have shown that the reaction was catalyzed in the presence of buffers capable of acting as an acid. This result indicated that the rate-determining step of the acid-catalyzed reaction involved proton transfer and not protonation of the substrate prior to the rate determining step, as shown in Scheme 7. This discovery leads to new issues.

Two kinetically equivalent mechanisms can be proposed where proton transfer occurs in the rate-determining step. In Scheme 8, a general-acid catalyzed mechanism is shown where rate-limiting proton transfer, from acid in solution, to the amide portion of the carbinolamide occurs as the protonated aldehyde departs. The kinetic expression for the mechanism shown in Scheme 8 is shown in equation 3.

Scheme 8: General-Acid Catalyzed Mechanism

Alternatively, a specific-acid followed by general-base catalyzed mechanism can also be proposed (see Scheme 9). Like the mechanism shown in Scheme 7, the carbinolamide is protonated on the amide portion to produce the protonated intermediate **12**. The rate-limiting step involves decomposition of **12** through a general-base catalyzed deprotonation of the hydroxyl group concurrent with breakdown to form the aldehyde and a tautomer of the amide. The kinetic expression for the mechanism (see eq. 4), shown in Scheme 9, is kinetically equivalent to that for the mechanism in Scheme 8. As a result, the actual mechanism by which the acid catalyzed reaction occurs requires further investigation.

Scheme 9: Specific-Acid Followed by General-Base Catalyzed Mechanism

$$k_{\text{obsd}} = \frac{k_1 k_2 [H^+][A^-]}{k_{-1} + k_2 [A^-]} = \frac{k_1 k_2 K_{\text{HA}} [\text{HA}]}{k_{-1} + k_2 [A^-]} = \frac{k_1 k_2 K_{\text{HA}} [\text{HA}]}{k_{-1}} = k_{\text{HA}} [\text{HA}] \quad (4)$$

The discovery of a reaction for the decomposition of carbinolamides where proton transfer is rate-limiting opens the possibility for enzymatic catalysis. The specific-base mechanism for the hydroxide-dependent reaction has breakdown of the conjugate base of the carbinolamide as the rate-limiting step. Enzymes have been shown to be very efficient at catalyzing proton transfer reactions, as such; the acid-catalyzed reaction would appear to be a more attractive mechanism for the enzyme. This outcome does not mean that PAL utilizes an acid-catalyzed mechanism during catalysis of the breakdown of

carbinolamides but simply that it is, perhaps, an attractive option. Many questions remain with regard to the exact mechanism which PAL utilizes. For example, there is still little understanding of the role of metal ions in the catalytic cycle of PAL.⁴⁹⁻⁶¹

Previous Metal Catalysis Studies:

It has been shown that a Zn^{2+} atom is essential for bioactivity of PAL although its role; structural or catalytic, is arguable.^{51,62} Calcium(II) and iron(III) have also been found in a purified PAL protein and their role in catalysis is also unknown. Studies where Zn^{2+} has been removed from the active enzyme, using EDTA, have shown that the enzyme loses activity. The reintroduction of Zn^{2+} and other metal-ions have been shown to reactivate the enzyme, providing evidence that the metal-ion was critical for catalysis. If the metal-ion plays a direct role in catalysis, two primary catalytic routes can be proposed: 1) the metal could bind a hydroxide and the complex could provide a catalytic base (Scheme 10) to deprotonate the carbinolamide and 2) the metal could bind to the carbinolamide and act as an electrophilic catalyst (Scheme 11).³⁸ The proposal that Zn^{2+} could have a direct catalytic role was supported by studies published by Takahashi that showed that Zn^{2+} and other metals catalyzed the reaction of carbinolamides in aqueous solution.³⁸ This has been confirmed by studies within our own group.^{63,64}

Scheme 10: Carbinolamide Breakdown

Scheme 11: Transfer of Charge via Coordination with Zinc

Takahashi and coworkers concluded that the metal catalysis occurred through a general base catalyzed deprotonation by a metal-bound hydroxide molecule through deprotonation of the hydroxyl group of the carbinolamide intermediate (Scheme 10).^{39,50,51} This mechanism contradicts the currently accepted hydroxide catalyzed mechanism for the aqueous reaction of carbinolamides which involves specific-base catalysis followed by rate determining breakdown.^{36,38,40,43-45,65} As implied earlier, general-base catalysis for the aqueous reaction of carbinolamides has not been observed at intermediate *pH*-values.^{36,40,65}

Questions:

- How do the α -hydroxyhippuric acid derivatives (**14**) react under acidic and basic conditions in the absence of the enzyme?
- Does the addition of a carboxylate group, which under basic conditions would be deprotonated, have an effect on the hydroxide-dependent mechanism?
- What is the effect of aromatic substituents on the acid and base catalyzed reactions of the α -hydroxyhippuric acid derivatives?

- d) Will the presence of the carboxylate group have an effect on the acid-catalyzed reaction?
- e) With the presence of the carboxylate group, will buffer catalysis in the acid-catalyzed region still be observed?
- f) What will the effect of the addition of metal-ions to the reaction solution have upon the acid and base-catalyzed reactions?

The initial portion of the project was the synthesis of several *para*-substituted derivatives of the parent compound α -hydroxyhippuric acid (**14**), a known substrate for PAL.⁴⁹ A method used by Bundgaard *et al.* and modified by previous students within the group was the primary method of synthesizing the compounds of interest.⁴³⁻⁴⁵

After synthesis of the α -hydroxyhippuric acid derivatives, the kinetic parameters for these compounds were determined in water spanning a *pH* range of 0-14 and *I* = 1.0M (KCl) at 25°C. The reactions were followed by UV-Vis spectroscopy using a Cary 1G spectrophotometer over most of the *pH*-rate profile. At *pH* of 4-6 (*pH* maintained using different buffers), the reactions were monitored using a Waters HPLC. At high hydroxide concentrations (*pH* > 11), the rates of reaction were too fast to be conveniently followed by UV-Vis spectroscopy so a stopped-flow apparatus was employed to obtain the rate constants at those hydroxide concentrations.

The data presented here will allow for an accurate comparison of experimental trends in rates of reactions to theoretical trends based on σ -values of the substituted molecules. It will enhance our understanding of the mechanistic criteria for the reaction of carbinolamides under aqueous conditions and potentially lead to a greater understanding of the enzymatic catalyzed reaction. In addition, the study of the metal-catalyzed reactions will broaden our understanding of the mechanistic criteria necessary for catalysis by an enzymatic system.

CHAPTER II

SYNTHESIS OF α -HYDROXYHIPPURIC ACID DERIVATIVES

Introduction

The primary goal of synthesizing α -hydroxyhippuric acid derivatives was to create a model compound for the carbinolamide intermediates generated in the PAM-catalyzed biological reaction. Once the derivatives were synthesized, the kinetic studies and the effect of substituents on the rate and mode of reaction will provide a greater understanding of the type of catalysis that can logically be proposed for PAM. The carbinolamide intermediate utilized in the PAM enzymatic reaction is from a glycine-extended peptide chain precursor. An important feature of the α -hydroxyhippuric acid derivatives is the fact that they contain the same modified glycine-extension off of the amide portion of the molecule as seen in the intermediates of the enzyme catalyzed reaction. Up to this point none of the carbinolamides studied within the group have contained the carboxylate extension.

For the formation of the α -amidated peptides, PAM first oxidizes and then breaks down the glycine-extended carbinolamide to create the bioactive peptide and glyoxylate.^{33,34} It is the goal of this work to gain an understanding of effects of the carboxylate group on carbinolamide reactivity. We will investigate the aqueous reaction

of α -hydroxyhippuric acid derivatives as a function of pH . These derivatives are better structural analogs of the actual carbinolamide substrate in PAM (in fact, α -hydroxyhippuric acid is a known substrate for the enzyme).⁴⁹ Also, studying α -hydroxyhippuric acid and various substituted derivatives will provide an understanding of electronic effects on carbinolamide reactivity.

Herein, a detailed synthetic route to synthesizing α -hydroxyhippuric acid derivatives will be described after which the rates of the hydroxide-independent, hydroxide-dependent, water reaction, and acid-catalyzed reactions for the selected carbinolamides will be determined. Studying these types of carbinolamides will help to support or not support what is currently known about the mechanisms of this functionality in an aqueous environment (general-acid, specific acid followed by general-base catalysis).

Experimental

Materials

All melting points reported were obtained using a Mel-Temp apparatus and are uncorrected. The 1H and ^{13}C -NMR spectra reported were obtained by dissolving the compound in deuterated DMSO (2.50 ppm) at 25°C using a Varian Mercury 500MHz spectrometer. All benzamides and carbinolamides used were synthesized in lab. Glyoxylic acid monohydrate was purchased from Acros Organics. All solvents were reagent grade. The chemicals used below were reagent grade and used without purification unless otherwise stated.

Synthesis

Benzamide Derivatives.

X	
H	14
CH ₃	15
OCH ₃	16
N(CH ₃) ₂	17
NO ₂	18
Cl	19
Br	20

The benzamide derivatives were synthesized (with the exception of 4-*N,N*-(dimethylamino)benzamide), using a procedure described in Vogel, by reacting the selected benzoic acid (~10.0g) with a molar equivalent of PCl₅ (Mallinckrodt Chemical) to produce the acyl chloride.⁶⁶ Once the formation of the acid chloride was complete, and the POCl₃ side product had been removed from the reaction vessel by vacuum distillation, ammonium hydroxide was added to the reaction vessel.⁶⁴ The ammonium hydroxide was added in a drop wise manner and the resulting mixture was allowed to stir for ~2 hours.⁶⁶ The crude product was isolated by vacuum filtration and purified by

recrystallization from an appropriate solvent.

The 4-*N,N*-(dimethylamino)benzamide compound was synthesized *via* the oxime derivative. The reaction was initiated by dissolving 7.490 g of hydroxylamine hydrochloride, 7.644 g of potassium acetate, and 7.496 g (0.050245 mol) of 4-*N,N*-(dimethylamino)benzaldehyde (Sigma-Aldrich) in 50 mL DI-water followed by gentle heating and stirring.⁶⁷ Once the reagents were dissolved, 50 mL of 95% ethanol was added to the solution and the mixture was allowed to sit at room temperature for approximately 10 minutes.⁶⁷ The formation of the oxime could be observed by the appearance of a white solid which was isolated by vacuum filtration and purified by recrystallization from water.⁶⁷ After recrystallization of the oxime and drying of the crystals under vacuum, 4-*N,N*-(dimethylamino)benzaldoxime was recovered.⁶⁸

The oxime of 4-*N,N*-(dimethylamino)benzaldehyde was then added to 25 mL boron trifluoride-acetic acid complex (98%) that had previously been heated to 115°C, for 10 minutes.⁶⁹ This served to convert the *syn*-aldoxime to the more reactive *anti*-aldoxime.⁶⁹ After the 10 minute reaction period the flask was cooled and 100 mL of 6M KOH was added to the reaction solution over 30 minutes, with continued cooling. As the reaction proceeded, the amide product precipitated out of solution and, after the reaction was complete, the product was isolated *via* vacuum filtration.⁶⁹ The amide was purified by recrystallization from methanol. Hot gravity filtration of the recrystallization solution was required to remove solid impurities.

Benzamide (14)

The melting point was found to be 125-127°C (literature melting point = 128°C).⁷⁰ ¹H-NMR 500MHz (DMSO): δ 7.35 (bs, 1H), 7.45 (t, 2H), 7.5 (t, 1H), 7.85 (d, 2H), 7.95 (bs, 1H).

4-Methylbenzamide (15)

The melting point was found to be 160-162°C (literature melting point = 160°C).⁷¹ ¹H-NMR 500MHz (DMSO): δ 2.35 (s, 3H), 7.25 (d, 2H), 7.25 (bs, 1H), 7.8 (d, 2H), 7.9 (bs, 1H).

4-Methoxybenzamide (16)

The synthesis of **16** was accomplished utilizing the method as described above, using 10 g of *p*-anisic acid (0.6572 mol) and a molar equivalent of PCl₅. After isolation and purification, 5.668 g was isolated corresponding to a 57% yield. The melting point was found to be 163-164°C (literature melting point = 167°C).⁷⁰ ¹H-NMR 500MHz (DMSO): δ 3.8 (s, 3H), 7.0 (d, 2H), 7.15 (bs, 1H), 7.8 (bs, 1H), 7.85 (d, 2H).

4-*N,N*-(Dimethylamino)benzamide (17)

The synthesis of **17** was accomplished using the method as described above. After isolation and purification, 3.125 g was isolated corresponding to a 37.8% yield. The melting point was found to be 208-210°C (literature melting point = 208°C).⁶⁹ ¹H-NMR 500MHz (DMSO): δ 7.35 (bs, 1H), 7.45 (t, 2H), 7.5 (t, 1H), 7.85 (d, 2H), 7.95 (bs, 1H).

4-Nitrobenzamide (**18**)

The melting point was found to be 198-200°C (literature melting point = 200°C).⁷² ¹H-NMR 500MHz (DMSO): δ 7.7 (bs, 1H), 8.1 (d, 2H), 8.25 (bs, 1H), 8.3 (d, 2H).

4-Chlorobenzamide (**19**)

The synthesis of **19** was accomplished using the general method described earlier. The compound 4-chlorobenzoic acid (10.003g, 0.0639mol) was used along with a molar equivalent of PCl₅. After isolation and purification, 1.618 g was isolated, corresponding to a 16% yield. The melting point was found to be 178-180°C (literature melting point = 178°C).⁷⁰ ¹H-NMR 500MHz (DMSO): δ 7.7 (bs, 1H), 8.1 (d, 2H), 8.25 (bs, 1H), 8.3 (d, 2H).

4-Bromobenzamide (**20**)

The synthesis of **20** was accomplished using the method as described above. The compound 4-bromobenzoic acid (10.0 g, 0.049746 mol) was used along with a molar equivalent of PCl₅. After isolation and purification, 8.726 g was isolated corresponding to a 87.6% yield. The melting point was found to be 182°C (literature melting point = 191°C).⁷⁰ ¹H-NMR 500MHz (DMSO): δ 7.7 (bs, 1H), 8.1 (d, 2H), 8.25 (bs, 1H), 8.3 (d, 2H).

α -Hydroxyhippuric Acid Derivatives.

X	
H	21
CH ₃	22
OCH ₃	23
N(CH ₃) ₂	24
NO ₂	25
Cl	26

α -Hydroxyhippuric Acid (**21**)

The synthesis of **21** was accomplished by dissolving benzamide (2.00 g, 0.0165 mol) in 30 mL acetone.⁴³⁻⁴⁵ Then, a molar equivalent of glyoxylic acid monohydrate was added to the round bottom flask.⁴³⁻⁴⁵ The mixture was heated under reflux for 24 hours. After 24 hours, the solution was allowed to cool to room temperature and then placed in the freezer to complete crystallization. The crystals were collected *via* vacuum filtration and purified by recrystallization in acetone. Once dried, 1.90 g of α -hydroxyhippuric acid was obtained (59% yield). The melting point was found to be 212°C (literature melting point = 211°C).⁷³ ¹H-NMR 500MHz (DMSO-d₆): δ 5.6 (d, 1H), 6.35 (bs, 1H), 7.45 (t, 2H), 7.55 (t, 1H), 7.9 (d, 2H), 9.2 (d, 1H), 12.85 (bs, 1H). ¹³C-NMR 400MHz (DMSO-d₆): δ 72.2, 127.5, 128.3, 131.6, 133.6, 166.2, 171.8.

4-Methyl- α -hydroxyhippuric Acid (22)

The synthesis of **22** was accomplished by using the synthesis described above in **21** by dissolving 4-methylbenzamide (2.00 g, 0.0146 mol) in 40 mL acetone. Once isolated, 1.13 g of 4-methyl- α -hydroxyhippuric acid was obtained (37% yield). The melting point was found to be 211°C. $^1\text{H-NMR}$ 500MHz (DMSO- d_6): δ 2.35 (s, 3H), 5.6 (d, 1H), 6.3 (bs, 1H), 7.3 (d, 2H), 7.85 (d, 2H), 9.15 (d, 1H), 12.7 (bs, 1H). $^{13}\text{C-NMR}$ 400MHz (DMSO- d_6): 20.9, 71.6, 127.5, 128.8, 130.8, 141.5, 165.8, 171.5.

4-Methoxy- α -hydroxyhippuric Acid (23)

The synthesis of **23** was accomplished by using the synthesis described above in **21** by dissolving 4-methoxybenzamide (0.506 g, 0.00333 mol) in 15 mL acetone. Once isolated, 0.301 g of 4-methoxy- α -hydroxyhippuric acid was obtained (40% yield). The melting point was found to be 224-227°C. $^1\text{H-NMR}$ 500MHz (DMSO): δ 3.85 (s, 3H), 5.1 (d, 1H), 6.3 (bs, 1H), 7.0 (d, 2H), 7.9 (d, 2H), 9.1 (d, 1H), 12.8 (bs, 1H). $^{13}\text{C-NMR}$ 400MHz (DMSO- d_6): δ 55.3, 71.6, 113.5, 126.5, 129.4, 161.82, 167.35, 171.5.

4-*N,N*-(Dimethylamino)- α -hydroxyhippuric Acid (24)

The recrystallization of **24** was carried out using a few mL of cold acetone followed by vacuum filtration. Once isolated, 0.218 g of 4-*N,N*-(dimethylamino)- α -hydroxyhippuric acid was obtained. The melting point was found to be 232°C. $^1\text{H-NMR}$ 500MHz (DMSO): δ 2.55 (s, 6H), 5.6 (d, 1H), 6.7 (d, 2H), 7.75 (d, 2H), 8.8 (d, 1H), 12.6 (bs, 1H). $^{13}\text{C-NMR}$ 400MHz (DMSO- d_6): δ 71.5, 110.7, 120.1, 128.86, 128.93, 152.3, 165.7, 171.7.

4-Nitro- α -Hydroxyhippuric Acid (25)

The synthesis of **25** was accomplished by using the synthesis described above, for **21**, by dissolving 4-nitrobenzamide (2.044 g, 0.00333 mol) in 20 mL acetonitrile. Once isolated, 1.185 g of 4-nitro- α -hydroxyhippuric acid was obtained (40% yield). The melting point was found to be 210°C. $^1\text{H-NMR}$ 500MHz (DMSO): δ 5.8 (t, 1H), 8.1 (d, 2H), 8.35 (d, 2H), 8.7 (d, 1H), 9.6 (d, 1H), 13.0 (bs, 1H). $^{13}\text{C-NMR}$ 400MHz (DMSO- d_6): δ 56.6, 123.57, 128.97, 138.84, 149.26, 164.49, 169.50.

4-Cl- α -Hydroxyhippuric Acid (26)

The recrystallization of **26** was carried out using a few mL of cold acetonitrile followed by vacuum filtration. Once isolated, 1.85 g of 4-chloro- α -hydroxyhippuric acid was obtained. The melting point was found to be 228-230°C. $^1\text{H-NMR}$ 500MHz (DMSO): δ 5.6 (d, 1H), 6.5 (bs, 1H), 7.55 (d, 2H), 7.95 (d, 2H), 9.3 (d, 1H), 12.8 (bs, 1H). $^{13}\text{C-NMR}$ 400MHz (DMSO- d_6): δ 71.7, 128.26, 129.36, 132.38, 136.42, 164.93, 171.27.

Results and Discussion

Synthesis of Benzamide Derivatives

The synthesis of the benzamide derivatives to be used for production of α -hydroxyhippuric acid compounds was achieved by two methods: firstly; by generating the oxime⁶⁵ and subsequent reaction to produce the amide and also, by generating the acyl chloride with subsequent treatment with NH_4OH (Scheme 12).⁶⁶ Two methods of generating the acyl chloride were utilized; a) Phosphorus pentachloride produces the acyl chloride, however the byproduct, POCl_3 , has to be removed before the acid chloride can

be used. b) Thionyl chloride, which bypasses the purification issue during the production of the acyl chloride, however, is not generally used with aromatic carboxylic acids. Once the acyl halide was generated, ammonium hydroxide was added to produce a crude benzamide product.⁶⁶ The benzamide derivatives were purified *via* recrystallization from water.⁶⁶

Scheme 12: Synthesis of Benzamide Derivatives

Both thionyl chloride and PCl_5 , followed by addition of ammonium hydroxide, failed to yield any amide product when using 4-*N,N*-(dimethylamino)benzoic acid. It is unknown why the reaction failed to synthesize this particular amide. The alternative procedure involved converting 4-*N,N*-(dimethylamino)benzaldehyde into an oxime.⁶⁷ The 4-*N,N*-(dimethylamino)benzaldehyde was mixed in proportion with potassium acetate and hydroxylamine hydrochloride to yield the oxime product in good yield.⁶⁷ The product of this reaction was the *syn*-oxime but this isomer was converted to the more reactive *anti*-isomer by heating the compound with boron-trifluoride-acetic acid complex.⁶⁷ Potassium hydroxide was then added to the mixture which reacted with the oxime and formed 4-*N,N*-(dimethylamino)benzamide.⁶⁷

Hoffenberg and Hauser proposed two possible mechanisms for the transformation of the oxime into 4-*N,N*-(dimethylamino)benzamide (Scheme 13).⁶⁹ Both mechanisms contain the same nitrile intermediate. The first possibility is a Beckmann-type

rearrangement (Scheme 13; a) where a hydride shift is accompanied by the loss of hydroxide, to produce a protonated nitrile group.⁶⁹ To yield the amide functionality, loss of a proton from the nitrogen and hydrolysis of the nitrile must occur. The other possibility is the dehydration (Scheme 13; b) of the oxime to produce the nitrile directly, then is hydrolyzed to form the amide.⁶⁹

Scheme 13: Synthesis of 4-*N,N*-(Dimethylamino)benzamide

Synthesis of α -Hydroxyhippuric Acid Derivatives

In the past, carbinolamides were synthesized through a reversible process involving the amide and aldehyde precursor (Scheme 14).^{36,40,52,74} The majority of the reactions were performed in methanol with potassium carbonate as a catalyst (Scheme 15). The reaction had the benefit of simplicity, however also had many downfalls. The reaction required large amounts of formaldehyde to obtain acceptable yields and required column chromatography for purification.

Scheme 14: General Synthesis of Carbinolamides

Scheme 15: Previous Synthetic Pathway of *N*-(Hydroxymethyl)benzamide Derivatives

Studies by Bundgaard *et. al.* opened a new route to synthesis of α -hydroxyhippuric acid derivatives without the excessive purification steps and use of toxic reagents (i.e. formaldehyde).⁴⁵ The selected benzamide was dissolved in a minimum amount of solvent followed by addition of molar equivalent glyoxylic acid monohydrate (Scheme 16). The reaction was allowed to heat at reflux for at least 12 hours with a maximum of 24 hours. The resulting solution was cooled to room temperature and placed in the freezer to crystallize for a few hours. The crude product was then isolated by vacuum filtration and purified by recrystallization.

Scheme 16: Synthesis of α -Hydroxyhippuric Acid Derivatives

This method proved to be useful for the synthesis of a few α -hydroxyhippuric acid derivatives, giving acceptable yields. However, the production of many of the target

derivatives proved to be tedious and inefficient in most cases. In an attempt to broaden the synthetic utility of the Bundgaard method, modifications were made that led to the eventual use of acetonitrile instead of acetone for synthesis of 4-nitro- and 4-chloro- α -hydroxyhippuric acid derivatives. Purification was further complicated by each derivative requiring several recrystallizations to purify the hippuric acid derivatives. Attempts at acid-base extractions resulted in loss of the carbinolamide. We believe this is because the rate of breakdown of these molecules can be quick even in solutions that are weakly basic. The synthesis above (Scheme 12) did prove to be unsuccessful for several other derivatives even with use of different solvent systems.

Conclusions

Amide synthesis proceeded in a very straight forward manner with acceptable yields of the amide recovered. The *N,N*-dimethylamino derivative did require a more elaborate proceed, however, ultimately the synthesis used worked quite well.

While procedures existed for the synthesis of the α -hydroxyhippuric acid compounds, it was found that procedures had to be modified before all the target compounds could be produced in a high enough yield. Recrystallization was not an ideal method for the purification of the carbinolamide target molecules as evidence was found for substrate degradation during the recrystallization process. Thus, multiple attempts at performing a “gentle” recrystallization were necessary. The term “gentle” means that no excessive heating of the solution to get the carbinolamide dissolved was used. When heated too vigorously, it became obvious that compound degradation was occurring as the product isolated by recrystallization was less pure than before recrystallization.

CHAPTER III

KINETIC STUDIES OF THE AQUEOUS REACTION OF α -HYDROXYHIPPURIC ACID DERIVATIVES

Introduction

Presented here are the kinetic studies, in aqueous media, of α -hydroxyhippuric acid derivatives containing electron-donating or electron-withdrawing groups substituted on the aromatic ring. These compounds are within the carbinolamide family, however there are some key features that make them unique and therefore worthy of investigation. In addition, the parent compound, α -hydroxyhippuric acid, is a known substrate for the enzyme PAL.³³ Through the study of the parent compound and its derivatives we can gain a better understanding of the mechanistic breakdown of these molecules. These compounds are also considerably more reactive than those previously studied most likely due to the added carboxylate group that can be protonated or deprotonated depending upon the pH of the reaction solution.

One of the key goals of this study, which is in unison with other studies performed in the Nagorski group, is to understand the key mechanistic steps in the breakdown of carbinolamides over the entire pH -rate profile. The foci will be on the four distinct regions of the pH -rate profile: acid-catalyzed, water-catalyzed, hydroxide-dependent, and hydroxide-independent regions. Coupled to this was the goal of

understanding how structural changes to the carbinolamide will affect the reactivity of the molecules themselves. The α -hydroxyhippuric acid derivatives have greater reactivity and an added carboxylate group which could in turn show new forms of reactivity during the course of their reaction. For example, previous studies with *N*-(hydroxymethyl)benzamide derivatives (**27**) utilizing both electron-donating and electron withdrawing substituents on the aromatic portion of the compounds illustrated the fundamental reactivity of carbinolamides in solution.^{36,40,52} Typically, electron-withdrawing groups increase the rate of the hydroxide-dependent reaction and slow the rate of the acid-catalyzed reaction and the converse holds true for the electron-donating groups.^{36,40} However, the study of these compounds also generated data showing that general acid catalysis was occurring in the acidic portion of the *pH*-rate profile.⁵² This was a new form of reactivity and led to the conclusion that the acid catalyzed reaction was not a specific-acid catalyzed process as others had previously concluded.^{38,43-45} Additionally, it has been shown that **27**, bearing a strongly electron-donating group, shows a unique form of reactivity at high hydroxide where there is a different mechanistic process which results in amide hydrolysis.^{68,76} The increased reactivity of the compounds studied here may lead to a greater understanding of these new reactivity patterns originally observed in the *N*-(hydroxymethyl)benzamide derivatives.

As previously mentioned in the introduction, two kinetically equivalent mechanisms can be proposed where proton transfer occurs in the rate-determining step.⁵² In Scheme 17, a general-acid catalyzed mechanism is shown where rate-limiting proton

transfer, from a proton source in solution, to the amide portion of the carbinolamide occurs as the protonated aldehyde departs. Alternatively, a mechanism involving specific-acid followed by general-base catalysis can also be proposed (see Scheme 18). Like the mechanism shown in Scheme 17, the carbinolamide is protonated on the amide portion to produce the protonated intermediate. The rate-limiting step involves decomposition through a general-base catalyzed deprotonation of the hydroxyl group concurrent with breakdown to form the aldehyde and a tautomer of the amide. As discussed earlier, these two mechanisms are kinetically equivalent and, as a result, the actual mechanism by which the acid catalyzed reaction occurs requires further investigation.

Scheme 17: General-Acid Catalyzed Mechanism

Scheme 18: Specific-Acid Followed by General-Base

The hydroxide-dependent reaction involves a specific-base catalyzed deprotonation of the hydroxyl group, generating the conjugate base of the carbinolamide (**14b**, Scheme 19). Then, in the rate limiting step, the carbinolamide undergoes an elimination to generate the glyoxylate product and an amidate leaving group. The amidate is subsequently protonated by solvent in a post-rate limiting step. This

mechanism is supported by a variety of observations but most powerfully by the absence of buffer-catalysis.^{40,63,64,68,76} In both hydroxide-dependent and acid-catalyzed mechanisms, the reaction results in a formation of an amide and glyoxylic acid.

Scheme 19: Generally Accepted Mechanism for the Hydroxide-Catalyzed Reaction of α -Hydroxyhippuric Acid Derivatives Under Aqueous Conditions

The objective of this project was to investigate the aqueous behavior of this unique class of carbinolamides, determine if the mechanistic conclusions from this study agree with previous studies, and delineate any other forms of reactivity that may be exhibited. Since α -hydroxyhippuric acid is a known substrate to the enzyme complex PAM,³³ learning more about this class of compounds could provide information about the mechanism utilized by the enzyme.

In previous studies, the carbinolamides that were investigated utilized aliphatic amide with aromatic aldehydes, aromatic amides with formaldehyde, and aromatic amides with aromatic aldehydes when studying the reactivity of the carbinolamides under aqueous reactions. The project discussed here consists of a new series of compounds, α -hydroxyhippuric acid derivatives (**14**), where there is an aromatic amide with an addition

of carboxylate group to the aldehyde.

Experimental

Materials

All chemicals, in these studies, were reagent grade and used without further purification unless specified otherwise. All carbinolamide starting materials were synthesized and purified as described in Chapter 2. Standard solutions ($\sim 0.1\text{M}$) of the α -hydroxyhippuric acid compounds were generated by dissolution of the compound in DMSO. Hydrogen chloride, potassium acetate, potassium hydrogen phosphate, potassium phosphate, potassium carbonate, potassium chloride, potassium pyrophosphate, and chloroacetic acid were used to generate buffer solutions at specific pH values. The water utilized to generate the solutions for the kinetic studies was house DI-water that was further purified *via* a Milli-Q water purification system. Standard 1.00 M potassium hydroxide solutions were prepared *via* dilution of a solution of $\sim 2.0\text{ M}$ potassium hydroxide. The exact molarity of the KOH solutions was determined through titrations to the phenolphthalein endpoint using a standardized 0.100 M HCl solution. The solution was then diluted to the desired 1.00 M KOH with subsequent titrations performed to verify final [KOH]. Standard 1.00 M HCl solutions were obtained in a similar manner. The final concentrations of the HCl solutions were determined by titration with standardized 0.100 M KOH solution to its phenolphthalein endpoint. Additionally, all buffer solutions used in these experiments used maintained $I = 1.00\text{ M}$ using KCl.

The following basic salts were used with their conjugate acids, acids with their conjugate base, or standard HCl solutions used to prepare buffered solutions of $I = 1.00$ at the pH indicated: chloroacetic acid (pH 2.703, 2.686, 3.050), potassium acetate (pH 4.124, 4.712, 5.138), potassium phosphate (pH 6.651, 7.737), potassium pyrophosphate (pH 8.584), and potassium bicarbonate (pH 9.712, 10.008, 11.038). In general the buffer concentrations were kept low (0.05 – 0.1 M) to minimize buffer catalysis in the more acidic regions of the pH -rate profile.

Kinetics

The rate of breakdown of the α -hydroxyhippuric acid derivatives into glyoxylic acid and benzamide varied widely between pH 0 to 14. To properly follow these reactions several instruments were used to determine the reaction rates as these values varied from seconds to several days.

Stopped-Flow Spectroscopy. The kinetic studies were observed at high hydroxide concentrations (pH 12 to 14) using a stopped-flow apparatus due to the rapid rates of reaction at these pH values. The stopped-flow used was an Olis spectrophotometer Cary-14 conversion, along with software supplied by Olis GlobalWorks to obtain k_{obsd} values. The temperature was maintained at 25°C. A typical experiment involved a 12 μL injection of a 0.1 M solution of the selected α -hydroxyhippuric acid derivative in DMSO into 10 mL of 1.00 M KCl with $I = 1.0\text{M}$ (KCl). Thus, reaction solutions had a final substrate concentration of approximately $2 \times 10^{-4}\text{M}$. Another solution was made with varying amounts of 1.00 M KOH and 1.00 M KCl. Those solutions made KOH concentrations from 0.01 – 1.0 M. The solutions were

temperature equilibrated at 25°C for at least 10 minutes prior to the commencing experiments. The two sets of solutions were mixed in a mixing chamber with equal volumes of solutions.

UV-Vis spectroscopy. The kinetic studies observed between pH 9 - 11.5 were followed employing a Varian Cary 1G UV-Vis spectrophotometer with a temperature controlled sample transport tray maintained at 25°C. The spectrophotometer and its temperature controller were operated *via* computer using Varian supplied software. Prior to injection of the carbinolamide being studied, the kinetic solutions were temperature equilibrated for ~10 to 15 minutes. Experiments were typically initiated using ~2-3 μL of a 0.100 M stock solution of the carbinolamide dissolved in DMSO, into 3 mL of a thermally equilibrated reaction solution in a 1 x 1 cm quartz cuvette, to yield a final concentration of $\sim 6.67 \times 10^{-5}$ - 1.00×10^{-4} M. The compounds studied were monitored *via* the change in absorbance at a specific wavelength between 240-330 nm (see Table 1) as a function of time. The observed rate constants were then obtained using the software supplied by Varian. The pH of the kinetic solutions were measured after the reaction was completed *via* standardized combination glass electrode attached to an Accumet Model 15 pH-meter from Fisher Scientific. The kinetic experiments were performed using at least 3 trials and then averaged to obtain the observed rate at the specific pH.

Table 1: Wavelengths used in UV-Vis Studies of the Aqueous Reaction of α -Hydroxyhippuric Acid Derivatives, 25°C, $I= 1.0$ M (KCl).

X
		4-OCH ₃
		4-CH ₃
		4-H
		4-N(CH ₃) ₂
		4-Cl
Compound		$\lambda_{\text{max}}(\text{nm})$
4-Methoxy- α -hydroxyhippuric acid	23	260
4-Methyl- α -hydroxyhippuric acid	22	252
α -Hydroxyhippuric acid	21	240
4- <i>N,N</i> -(Dimethylamino)- α -hydroxyhippuric acid	24	328
4-Chloro- α -hydroxyhippuric acid	26	255

HPLC Kinetic Studies. The kinetic experiments between *pH* 0-9 were too slow to follow conveniently with the UV-Vis spectrophotometer. For these experiments, the reaction was followed by means of an HPLC instrument. The HPLC employed consisted of two Waters 515 pumps, a Waters Guard-Pak with C-18 inset, a Waters radial compression module with C-18 reverse phase column, a Waters 996 photodiode array detector, and a Waters 717_{plus} Autosampler. All instrumentation was controlled remotely *via* computer equipped with Empower Pro software.

A general HPLC experiment commenced with the ~ 3 μL injection of a stock solution of the selected α -hydroxyhippuric acid derivative in DMSO (0.100 M) into 3 mL

of the reaction solution (with varying buffer concentration) to yield the substrate concentration of $\sim 6.67 \times 10^{-5}$ - 1.00×10^{-4} M. The samples were thermally equilibrated throughout the reaction period in a Waters 717_{plus} Autosampler maintained at 25 °C. The reaction progress was monitored by periodic injection of 100 μ L volumes of the reaction solution. The ratio of product to starting material was followed by the observation of the disappearance of the carbinolamide by monitoring the peak area (A_{car}) and the subsequent appearance of the amide (A_{amide}) as a function of time (sec). The Δt was determined for each experiment by taking the difference between the time of the first injection and the time of the injection of interest.

$$\ln \left(\frac{A_{car}}{(A_{car} + ((R_x)(A_{amide \text{ or ald}}))} \right) = k_{obsd} t \quad (5)$$

The observed rates of the reaction utilizing the HPLC were calculated using equation (5). The areas observed for the amide were statistically corrected for differences in the molar absorptivities between the carbinolamide and the amide. The correction factor (R_x) was determined by following the reaction by HPLC for ~ 2 -5 half-lives and determining the differences in the total area changes between the carbinolamide and the amide (R_x values shown in Table 2). The correction factor (R_x) corrects for differences in the molar extinction coefficients between the observed areas for the amide and the losses observed in the area of the carbinolamide peak. The observed rate of the reaction was extrapolated as the slope of the natural log of the corrected fraction of starting material vs. time. For experiments at pH 2 to 6, the observed rate was determined using an initial rates technique.⁷⁵ Utilizing this method, the first ~ 3 -5% of the reaction was followed and

the data were plotted similarly to the other experiments collected on the HPLC.

Following the completion of each reaction, the *pH* was measured. No significant changes in the *pH* were observed during the course of these studies.

Table 2: Calculated Correction Factors with Solvent Systems and Wavelengths used for the HPLC Studies of the Aqueous Reaction of α -Hydroxyhippuric Acid Derivatives at 25 °C, *I* = 1.0 M (KCl)

Compound ^{a,b}		$\lambda_{\text{HPLC}}(\text{nm})$	Correction ^c Factor (<i>R_x</i>)	Solvent ^d System
4-Methoxy- α -hydroxyhippuric acid	23	240	2.22	70/30
4-Methyl- α -hydroxyhippuric acid	22	240	2.27	70/30
α -Hydroxyhippuric acid	21	240	1.92	80/20
4- <i>N,N</i> -(Dimethylamino)- α -hydroxyhippuric acid	24	240	2.70	80/20
4-Chloro- α -hydroxyhippuric acid	26	240	1.86	70/30

^aExperiments were performed in H₂O at 25 °C, *I*=1.0M (KCl) with substrate concentrations of $6.67 \times 10^{-5} - 1 \times 10^{-4}$ M. ^bInjections of 100 μL into the HPLC system utilizing C-18 reverse phase column. ^cDetermination of the correction factor for the carbinolamide vs. benzamide were over 2-5 half-lives and determined by correlating changes in the observed area of the carbinolamide vs. benzamide. ^dSolvent system refers to a combination mixture of 0.01M K₂HPO₄ and pure HPLC grade methanol.

Results and Discussion

Kinetics

The α -hydroxyhippuric acid derivatives studied varied by substituents placed in the *para*-position of the aromatic ring. The derivatives included both electron-donating and withdrawing groups. The 4-nitro- α -hydroxyhippuric acid derivative kinetics could not be studied for reasons that have not yet been fully elucidated, so the results presented here will focus on the other five derivatives. Previous studies performed by the Nagorski

group show that as the substituents, placed on the amide portion of the carbinolamide, becomes increasingly electron-donating the rate of the hydroxide-dependent reaction decreases.^{40,63,64,68,76} On the other hand, in the acid-catalyzed region the more electron-withdrawing a substituent yielded the slower the rate of reaction.^{40,52,63,64,68,76}

To investigate the mechanisms in aqueous media in these class of compounds the rate constants were determined in water, at 25°C, $I = 1.0$ KCl. Stopped-flow spectrophotometer, UV-Vis spectrophotometer, and HPLC apparatus were used to determine the rate constants of the α -hydroxyhippuric acid derivatives, by following the breakdown of the carbinolamide into the product amide. For the reactions between $pH = 2$ to 6, initial rates method was used. Spectrophotometric rate constants were determined by following the rate of absorbance *vs.* time. The HPLC studies determined the rates by following the differences in the total area change for the carbinolamide (A_{car}) *vs.* the amide (A_{amide}). In Figure 5, the dependence of the $\log k_{obsd}$ *vs.* pH of the reaction solution in H_2O , $I = 1.0M$ KCl, at 25°C.

Figure 5: pH-Rate Profile for Aqueous Reaction of 4-Chloro- α -hydroxyhippuric Acid Derivatives in H₂O at 25°C, and $I = 1.0\text{M}$ (KCl).

As has been discussed in previous sections of the thesis, the pH -rate profiles of the α -hydroxyhippuric acid compounds is similar to previously studied carbinolamides.^{40,52,63,64,68,76} That is, there are four distinct regions of the pH -rate profile. There is a hydroxide-dependent region that becomes hydroxide independent at high pH . There is a water catalyzed region between $pH = 2$ to 6 which is a much broader than any of the water-catalyzed regions observed for other carbinolamides that have been studied. Finally, there is the acid-catalyzed region. Prior studies with α -hydroxyhippuric acid by Bundgaard *et al.*⁷⁷ did not detect the presence of this reaction, but for all derivatives of **14** investigated, the acid-catalyzed reaction was observed. Each kinetic region will be discussed separately.

Initial studies of the derivatives of **14** focused on the hydroxide-dependent and -independent regions. All of the compounds investigated, with the exception of 4-(*N,N*-dimethylamino)- α -hydroxyhippuric acid, showed good pseudo-first order kinetics over the pH -range. However, extensive product studies for the various compounds have not been completed for this region of the pH -rate profile. A variety of techniques were employed to study the hydroxide region of the pH rate profile including stopped-flow, UV-Vis, and HPLC spectrometry.

Hydroxide-Dependent and -Independent Region

One of the aspects of the α -hydroxyhippuric acid derivatives that make them unique with respect to other carbinolamides, that have been investigated, is the ionizable proton of the carboxylate group of **14**. The pK_a of α -hydroxyacetic acid is 3.83 and, if it

Figure 6: Effect of Increasing Hydroxide Concentration of k_{obsd} on the Breakdown of α -Hydroxyhippuric Acid Derivatives in H_2O , at 25°C , and $I = 1.0\text{M}$ (KCl). (●) 4-chloro- α -hydroxyhippuric acid; (▼) α -hydroxyhippuric acid; (◆) 4-methyl- α -hydroxyhippuric acid; (■) 4-methoxy- α -hydroxyhippuric acid; (▲) 4-(N,N -dimethylamino)- α -hydroxyhippuric acid.

is assumed the pK_a of the carboxylate group in the derivatives of **14** are similar, then the carboxylate group of **14** would be deprotonated over the entire hydroxide-dependent region. (It would be expected that the substituents on **14** would have almost no effect on the pK_a of the carboxylate group as it has been shown that the substituents have only very small effects on the pK_a of the hydroxyl group of the *N*-(hydroxymethyl)benzamide and *N*-(hydroxybenzyl)benzamide.)^{40,63,64,68,76} What effect will the presence of the carboxylate have on the reactivity of the derivatives of **14**? The ionized carboxylate group could have an effect on the pK_a of the hydroxyl group of the carbinolamide due to their proximity. However, the addition of the negatively charged carboxylate should also provide inductive assistance to the departure of the benzamidate leaving group (see Scheme 19).

Shown in Figure 6 is the plot of k_{obsd} vs. the $[\text{HO}^-]$ for the five compounds that were studied. From Figure 6, at low $[\text{HO}^-]$ there is a first-order dependence on k_{obsd} which becomes hydroxide independent at higher $[\text{HO}^-]$. These plots are similar to k_{obsd} vs. $[\text{HO}^-]$ plots observed previously for *N*-(hydroxymethyl)benzamides, *N*-(hydroxybenzyl)acetamides, and *N*-(hydroxybenzyl)benzamides. In addition, in those studies performed below *pH* values of 12, buffers were used to maintain the *pH* of the reaction solution during the kinetic experiment, and no evidence for the presence of buffer-catalysis was observed meaning that proton transfer was not occurring in the rate-limiting step. The shape of the plot, in Figure 6, and the absence of buffer-catalysis provide strong evidence for a reaction mechanism, for the derivatives of **14**, involving specific-base catalysis. Such a process has the base extract a proton from the substrate

and it is the deprotonated substrate that subsequently undergoes rate-limiting breakdown. Thus, the rate of the reaction is dependent on the concentration of the base, but the rate-limiting step does not involve proton transfer. Under these circumstances, if the proton being removed is sufficiently acidic, saturation kinetics can be observed.

From Figure 5, the hydroxide-dependent mechanism dominates at pH values above 6. As stated above, the substrate will be fully deprotonated at pH values of ~ 5 , assuming a pK_a of ~ 3.8 for the carboxylate (based upon pK_a for α -hydroxyacetic acid). However, based upon the appearance of Figure 6 and the lack of buffer-catalysis, in addition to other facts that will be subsequently discussed, the mechanism of hydroxide-dependent breakdown of the α -hydroxyhippuric acid derivatives occurs by a specific-base catalyzed reaction (Scheme 19). The first step involves ionization of the carboxylic acid group to generate **14a**. At a specific pH , an equilibrium between **14a** and **14b** will be established that will be based upon the pK_a of the hydroxyl group of the carbinolamide. As the pH of the reaction solution increases, the equilibrium will shift towards **14b** thereby increasing the concentration of what is thought to be the intermediate that undergoes rate-determining breakdown.

The rate-determining step of the reaction is the breakdown of **14b** by the departure of the benzamidate leaving group and the formation of glyoxalate. Due to the observed dependence of k_{obsd} on the $[HO^-]$, it is not necessarily apparent that it is the breakdown of **14b** that is rate-limiting. However, it is when the reaction becomes hydroxide independent that the true nature of the reaction becomes more apparent. As the

pH of the reaction increases, the equilibrium between **14a** and **14b** will continue to shift towards **14b** until the K_a of the hydroxyl group is reached. At that point further increases in hydroxide have no effect as the substrate has been fully deprotonated and the only form in solution is **14b**.

$$(k_{\text{obsd}})^{\text{HO}} = k_1 \frac{K_a [\text{HO}^-]}{K_w + K_a [\text{HO}^-]} \quad (6)$$

$$(k_{\text{obsd}})^{\text{HO}} = k_1 \frac{K_a [\text{HO}^-]}{K_w} = k'_1 [\text{HO}^-] \quad (6a)$$

Table 3: Rate Constants for the Hydroxide-Independent and Dependent Reactions of α -Hydroxyhippuric Acid Derivatives and their $\text{p}K_a$'s in Water at 25°C, $I=1.0$ M (KCl).

X			
		4-OCH ₃			
		4-CH ₃			
		4-H			
		4-N(CH ₃) ₂			
		4-Cl			
Compound		k_I (s ⁻¹)	K_a (M)	$\text{p}K_a$	k'_1 (M ⁻¹ s ⁻¹)
4-N(CH ₃) ₂	24	0.650	5.14×10^{-14}	13.27	2.57
4-OCH ₃	23	0.664	8.55×10^{-14}	13.07	3.43
4-CH ₃	22	0.773	5.31×10^{-14}	13.27	3.47
4-H	21	0.986	4.96×10^{-14}	13.30	3.93
4-Cl	26	1.361	5.55×10^{-14}	13.25	5.09

Figure 7: Hammett Plot of Apparent Second-Order Rate Constants (k'_1) for the Hydroxide Reaction of the α -Hydroxyhippuric Acid Derivatives in Water, at 25°C and $I = 1.0\text{M}$ (KCl).

Apparent Second-Order Rates (k'_1): Using the mechanism outlined in Scheme 19, an equation for the observed rate of the reaction (k_{obsd}) can be derived (see equation 6). As is apparent from Figure 6, at low $[\text{HO}^-]$ there is a linear correlation between the $[\text{HO}^-]$ and k_{obsd} . Under these conditions, $K_a[\text{HO}^-]$ is small compared to K_w and the equation simplifies as shown in equation 6a. This leads to the linear portion of the pH -rate profile and is the often reported k'_1 value (apparent second-order rate constant for the hydroxide reaction). The apparent second-order rate constants are obtained from the linear portion of the k_{obsd} vs. $[\text{HO}^-]$ and are reported in Table 3. The apparent second-order rate constants show that as electron-withdrawing groups are added to the aromatic ring of the amide portion of the carbinolamide, the rate of reaction increases relative to those bearing electron-donating groups. It is apparent when comparing equations 6 and 6a that the k'_1 value incorporates two variable constants and it is therefore difficult to assess what the real meaning of the observed changes in k'_1 indicate.

Shown in Figure 7 is the Hammett plot of the apparent second order rate constants for the hydroxide reaction. The plot is linear over the range of compounds studied indicating that the same mechanism was being utilized by all the compounds. The ρ -value of the plot was 0.26, indicating that there is a build of negative charge in the rate-limiting step of the reaction. However, it is difficult to assess the nature of this charge buildup. For example, increases in the K_a of the hydroxyl group due to the electron-withdrawing nature of the substituent should lead to increases in k'_1 . However, the electron-withdrawing groups should also increase the ability of the leaving group to stabilize charge making it a better leaving group and increases in k_1 . Thus, no firm

Figure 8: Hammett Plot of K_a 's for the α -Hydroxyhippuric Acid Derivatives Studied in H_2O , at 25°C and $I = 1.0\text{M}$ (KCl)

conclusions with respect to the rate-limiting step can be made and it is important to complete the full hydroxide portion of the plot so that a nonlinear least squares analysis of the data can be performed which will allow both k_1 and K_a to be determined.

Dissociation Constant of the Carbinolamide Hydroxyl Group: The fit lines shown in Figure 6 are a result of the nonlinear least square fit of the data to equation 6 where K_a is the dissociation constant for the hydroxyl group of the carbinolamide, K_w is the dissociation constant for water and k_1 is the first-order rate for the decomposition of **14a** into glyoxylate and benzamide. The constants that were determined in this manner are listed in Table 3. Also listed in Table 3 are the pK_a values calculated using the K_a values reported.

As has been previously found in other studies, substituents attached to the aromatic ring on the amide side of the carbinolamide have only small effects on the acidity of the hydroxyl group of the carbinolamide.^{40,63,64,68,76} The data in Table 3 shows no trends based upon the substituents attached (see Figure 8 for Hammett plot of K_a values). A ρ -value of -0.005 for the Hammett plot of the K_a values for the hydroxyl group is shown in Figure 8. This result indicates that the substituents attached to the amidic portion of the carbinolamide are having essentially no effect. It would be anticipated that the electron-withdrawing chloro group would increase the acidity of the hydroxyl group relative to those α -hydroxyhippuric acid derivatives bearing electron-donating groups.

A similar lack of substituent effect was observed for the *N*-(hydroxymethyl)benzamide compounds⁴⁰ and a similarly weak trend was observed with

the *N*-(hydroxybenzyl)benzamide compounds.^{63,64} The only carbinolamides studied to date that exhibited significant substituent dependence were the *N*-(hydroxybenzyl)acetamide derivatives where the substituents were placed on the benzyl group. The greater proximity and shorter inductive effect pathway were believed to have led to this observation.⁷⁸

A possible explanation for the lack of significant substituent effect could be linked to the proximity of the carboxylate group. The *N*-(hydroxybenzyl)benzamide derivatives had pK_a 's of ~ 12.8 ;⁴⁰ *N*-(hydroxymethyl)benzamide derivatives had pK_a 's of ~ 13 ,⁶⁴ and the *N*-(hydroxybenzyl)acetamide derivatives had pK_a 's of ~ 12.9 .⁷⁸ The pK_a 's of the α -hydroxyhippuric acid derivatives (pK_a 's of ~ 13.3) are slightly higher than the other families of carbinolamides studied suggesting that the carboxylate does have a moderating effect on the acidity of the hydroxyl group. It is the insensitivity of the pK_a 's to the presence of the substituents that provides evidence that the substituents effect on the K_a of the hydroxyl group may be masked by the dominant effect of the carboxylate group.

Limiting Rate Constants (k_1): The most significant effect of the substituents, on the reactivity of the α -hydroxyhippuric compounds, occurs in the rate-limiting decomposition **14b** (k_1). The k_1 values determined by nonlinear least squares fit of the kinetic data to equation 6 are listed in Table 3. As electron-withdrawing groups are added to the amide portion of the **14**, the limiting rate of the reaction, under basic conditions, increases. This observation can be explained by the electron-withdrawing groups

Figure 9: Hammett Plot of k_1 for the α -Hydroxyhippuric Acid Derivatives Studied in H_2O , at 25°C and $I = 1.0\text{M}$ (KCl)

assisting in the stabilization of the negative charge being generated on the benzamidate group during the breakdown of **14b** in glyoxylate and the benzamide derivative.

The effect of the substituents can be more easily observed in Figure 9, which shows the Hammett plot of the substituent effects on k_1 . Two observations are immediately clear; a) The electron-withdrawing groups increase the rate of the limiting decomposition of the conjugate base of the carbinolamide. b) The 4-(*N,N*-dimethylamino)- α -hydroxyhippuric acid derivative does not fit on the plot with the four other compounds that were investigated.

The linear portion of the Hammett plot in Figure 9, shows that these four compounds react by a common mechanism. The slope or ρ -value of the data shown in Figure 9 was $\rho = 0.62$. The positive value of ρ indicates that negative charge is being generated, that can interact with the substituents, in the rate-limiting step of the reaction. It has been shown above that the hydroxyl group of the carbinolamide seems to be too far and/or too well insulated from the substituent groups for them to have a significant effect on the pK_a of the hydroxyl group. Given the accepted hydroxide-dependent mechanism (see Scheme 19), the substituents would be assisting in the stabilization of the negative charge that is being generated on the nitrogen of the amide during rate-limiting decomposition.

Very little data exists concerning the acidity of amides, specifically benzamides as related to this study. Available data suggests that the pK_a of protons on nitrogen can vary

from >19 for benzamide, to 15.85 for 4-nitrobenzamide.^{79,80} Thus, it is not too surprising that the substituents would have a significant effect on the leaving group ability of the benzamides involved in this study. In the study of the *N*-(hydroxymethyl)benzamide compounds the ρ -value for the k_1 values was $\rho = 0.67$,^{36,40} and for the *N*-(hydroxybenzyl)benzamide compounds the ρ -value was $\rho = 0.65$.^{63,64} In both these studies benzamides were the leaving groups in the study and it was somewhat surprising to see such a similar sensitivity to the nature of the leaving group in the two different families of carbinolamides.

Why was the similarity of the ρ -values in the two previous studies surprising? The breakdown of **14b** or the conjugate base of any carbinolamide has two aspects. The first is the leaving group (amidate). The barrier to breakdown will be related to the nucleofugality of the amidate. The better the leaving group, the lower the barrier to product formation will be. Thus, the observed substituent dependence observed in the *N*-(hydroxymethyl)benzamide derivatives and the *N*-(hydroxybenzyl)benzamide derivatives. The addition of the electron-withdrawing substituents increases the leaving group ability of the benzamide and the reaction goes faster.

The second portion of the breakdown of the conjugate base of the carbinolamide is the carbonyl compound that is being generated. It is the electron donation from the oxygen that assists in the departure of the amidate leaving group. The stability of the carbonyl compound formed will also have a role in lowering the barrier to the reaction. In

addition, any substituents attached to this portion of the molecule will affect the barrier to reaction. Groups that are capable of donating electron-density will inductively assist in the departure of the leaving group. Studies with *N*-(hydroxybenzyl)acetamide derivatives, where substituents were attached to the carbonyl portion of the carbinolamide, showed a significant sensitivity to the type of substituent (ρ -value = -0.91).⁷⁸ In the case of the *N*-(hydroxybenzyl)acetamide derivatives electron-donating groups aided the departure of the amide leaving group by inductively providing electron density.

A ρ -value of 0.62 for the α -hydroxyhippuric acid derivatives is only marginally different from the other two studies that have been performed. The reaction appears to be slightly less sensitive to the substituent effects and this can be partially explained by the negative charge on the carboxylate providing some inductive electron assistance to the departure of the benzamide leaving group. The conclusion that can be reached from this analysis is that the α -hydroxyhippuric acid compounds show all characteristics of reacting in the same manner as those that have been previously studied. The anomalous compound, with respect to the α -hydroxyhippuric acid derivative, is 4-(*N,N*-dimethylamino)- α -hydroxyhippuric acid (**24**).

From Figure 9, it is apparent that the limiting rate for the hydroxide-dependent reaction (k_1) is larger than would be predicted based upon the experimental trend generated by the other α -hydroxyhippuric acid derivatives studied. The type of deviation from linearity observed in Figure 8 generally occurs when there is a change in the

mechanism of reaction occurring. What makes this observation somewhat unique was the observation that the apparent second-order rate constant plot was linear (see Figure 7). Coupling the results shown in Figures 7 and 9 indicates that at lower [HO⁻] the mechanism, for all compound studied, was the same. It was only at higher [HO⁻] that the mechanism of **24** deviated.

An observation of this type was previously observed for a series of *N*-(hydroxymethyl)benzamide derivatives bearing electron-donating groups.^{68,76} In this series of compounds, it was shown, by product analysis, that rapid amidic hydrolysis was occurring competitively with “normal” carbinolamide breakdown. A proposed mechanism for this reaction is shown in Scheme 20. The rationale provided to account for the onset of the competitive hydrolytic mechanism was based upon the electron-donating substituents reducing the leaving group ability in **Y**, which in turn led to the possibility of competitive reactions becoming energetically viable. The net result, in the study of the *N*-(hydroxymethyl)benzamide derivatives bearing electron-donating groups was that the limiting rates were faster than structurally analogous compound bearing electron-withdrawing groups, the same observation seen in Figure 8.

Scheme 20: Proposed Hydrolytic Mechanism for *N*-(Hydroxymethyl)benzamide Derivatives Bearing Electron-Donating Substituents

The exact nature of the mechanism for the hydrolytic reaction in *N*-(hydroxymethyl)benzamide must remain speculative as it is not the focus of the work presented here. However, the similarity of the observations, associated with the reaction of **24** at higher $[\text{HO}^-]$ and the *N*-(hydroxymethyl)benzamide derivatives, suggest that it may be undergoing a similar type of reaction. Experimental proof of hydrolytic products was not investigated at the time these experiments were being performed and remains a piece of work that needs to be further investigated.

Table 4: Rate Constants for the Water Reaction of α -Hydroxyhippuric Acid Derivatives in Water at 25°C, $I= 1.0$ M (KCl).

X
			4-OCH ₃
			4-CH ₃
			4-H
			4-N(CH ₃) ₂
			4-Cl
Compound		$k_{\text{HOH}} (\text{s}^{-1})$	
4-N(CH ₃) ₂	24	1.9×10^{-7}	
4-OCH ₃	23	2.0×10^{-7}	
4-CH ₃	22	2.1×10^{-7}	
4-H	21	1.60×10^{-7}	
4-Cl	26	1.0×10^{-7}	

Water-Catalyzed Reaction

Another interesting observation with regard to the α -hydroxyhippuric acid derivatives was the large region in the pH -rate profile that was dominated by the water reaction. For all other carbinolamides previously studied the water reaction dominates for 2 to 3 pH units. The α -hydroxyhippuric acid compounds have the water reaction dominating for ~ 4 (see Figure 5). This region extends from pH 2 to 6 and what is even more intriguing is the fact that the substrate is changing its ionization state in this region and there does not appear to be a significant observable effect on the reactivity.

The pK_a of glyoxylic acid is 3.32⁸¹ and the pK_a for α -hydroxyacetic acid is 3.83.⁸² The pK_a of α -hydroxyhippuric acid has not been reported but it would be anticipated that it would be closer to the 3.32 value than 3.83 of α -hydroxyacetic acid. However, if one studies the pH -rate profiles (see Figure 5 and Supplemental materials), the region does not undergo substantial rate changes that may be associated with a change in the ionization state.

The observed rates for the water reaction (k_{HOH}) are listed in Table 4 and, as with the pK_a data (see Table 3), no significant trend can be observed in the data. Compound **26** reacted the slowest of the compounds studied but it is difficult to assess the precision of the values reported in Table 4. Other problems arise when looking at the data reported in Table 4.

The mechanism by which the water reaction occurs is not known. It can be stated that water or the elements of water catalyze the breakdown of the carbinolamide. However, the mechanism is not known. Does the reaction occur *via* a specific-acid followed by general base mechanism, or does water catalyze the decomposition by some unknown mechanism.

Another consideration is that the acid-catalyzed reaction is known to involve general-acid catalysis (buffer catalysis, see earlier discussion) but the presence or absence of such catalysis was not studied in detail during the course of these studies. Thus, no conclusions concerning the nature of the water-catalyzed reaction can be tendered.

Acid-Catalyzed Region

One of the unique discoveries found during the investigation of the α -hydroxyhippuric acid derivatives was the presence of an acid-catalyzed reaction. Previous studies of α -hydroxyhippuric acid did not provide evidence for the presence of such a reaction but all of the α -hydroxyhippuric acid derivatives investigated clearly underwent such a reaction (see Figure 5 and Supplement Data). Unfortunately not enough rate constants at different [HCl] were collected to acquire a second-order rate constant for the acid-catalyzed reaction. Only 4-chloro- α -hydroxyhippuric acid had enough data collected to provide a second-order rate constant. The rate of acid-catalyzed reaction of **26** was $k_H = 8.5 \times 10^{-7} \text{ M}^{-1}\text{s}^{-1}$.

This is considerably slower than the acid-catalyzed reactions for *N*-(hydroxybenzyl)benzamide and *N*-(hydroxybenzyl)acetamide carbinolamide derivatives studied and approximately an order of magnitude slower than the *N*-(hydroxymethyl)benzamide derivatives studied. This could be due to the fact that the protonated carboxylate group would act as an electron-withdrawing group and slow the departure of the leaving group. Given the mechanistic ambiguity surrounding the acid-catalyzed reaction (see discussion earlier), it is difficult to discuss the effects that the substituents are having on the reactivity of the α -hydroxyhippuric acid derivatives. Coupling the lack of acid-catalyzed rate constants for the compounds under discussion to the lack of an accepted mechanism means no firm conclusions can be proposed.

Conclusions:

Presented herein are the results of the investigation of the *pH*-dependent aqueous reaction of a series of aromatic substituted α -hydroxyhippuric acid derivatives. The compounds undergo an acid and water catalyzed reaction, as well as a hydroxide-dependent reaction. The reaction rates between *pH* 0 and 14 were studied with attention given to the effect of added general-acid and general-base catalysts. A *pH*-rate profile can be seen in Figure 5 and, for the other compounds studied, the Supplemental Material.

An acid-catalyzed reaction was observed for all α -hydroxyhippuric acid derivatives studied. However, a lack of extensive detailed information made it difficult to shed any light of the mechanistic ambiguity surrounding the acid-catalyzed reaction.

The water-reaction was an important reaction pathway for the α -hydroxyhippuric acid derivatives and dominated over a much greater breadth of the *pH*-rate profile than for other families of carbinolamides that have been studied. Unfortunately, similar to other carbinolamides that have been previously studied, the water-catalyzed reactions were very slow, precluding the acquisition of large amounts of kinetic data focusing on this reaction. The rates for the water-catalyzed reaction do not show any obvious trends for the breadth of the compounds studied.

The hydroxide-dependent reaction was surprisingly typical, as compared to other carbinolamides studied. The hydroxide-dependent reaction occurs *via* a specific-base catalyzed deprotonation of the hydroxyl group of the carbinolamide followed by rate-limiting breakdown. At the *pH* of the hydroxide-dependent reactions were dominating, it

would be expected that the carboxylate group of α -hydroxyhippuric acid would be ionized. Thus, deprotonation of the hydroxyl group would mean that the compound would have a charge of -2. Ultimately, only small changes in the pK_a of the hydroxyl groups were noted relative to other carbinolamides that have been investigated.

It was the rate-limiting step where questions concerning the mechanism by how these compounds react. The compound bearing the *N,N*-dimethylamino substituent appeared to have a higher limiting rate than would be anticipated based upon the other compounds studied (nonlinear Hammett plot). In previous studies this observation was associated with a new mode of reactivity becoming competitive with “normal” carbinolamide breakdown. This mechanism was a rapid amidic hydrolysis pathway. Evidence for a similar pathway for the reaction of 4-(*N,N*-dimethylamino)- α -hydroxyhippuric acid has yet to be collected but this type of reactivity is suspected.

All evidence, for the remaining α -hydroxyhippuric acid derivatives, strongly points toward the hydroxide-dependent reaction occurring *via* a specific-base catalyzed mechanism. The compounds studied herein proved to react in a manner similar to other carbinolamides studied but also supplied surprises that will require further investigation to probe the reasons for these observed mechanistic anomalies.

Supplemental Material

Figure S1: pH-Rate Profile for Aqueous Reaction of 4-(*N,N*-dimethylamino)- α -hydroxyhippuric Acid Derivatives in H_2O at 25°C , and $I = 1.0\text{M}$ (KCl).

Figure S2: pH-Rate Profile for Aqueous Reaction of 4-Methoxy- α -hydroxyhippuric Acid Derivatives in H₂O at 25°C, and $I = 1.0\text{M}$ (KCl).

Figure S3: pH-Rate Profile for Aqueous Reaction of 4-Methyl- α -hydroxyhippuric Acid Derivatives in H₂O at 25°C, and $I = 1.0\text{M}$ (KCl).

Figure S4: pH-Rate Profile for Aqueous Reaction of α -Hydroxyhippuric Acid Derivatives in H_2O at 25°C , and $I = 1.0\text{M}$

Figure S5: Effect of Increasing Hydroxide Concentration of k_{obsd} on the Breakdown of 4-(*N,N*-dimethylamino)- α -hydroxyhippuric acid in H_2O , at 25°C , and $I = 1.0\text{M}$ (KCl).

Figure S6: Effect of Increasing Hydroxide Concentration of k_{obsd} on the Breakdown of 4-methoxy- α -Hydroxyhippuric acid in H_2O , at 25°C , and $I = 1.0\text{M}$ (KCl).

Figure S7: Effect of Increasing Hydroxide Concentration of k_{obsd} on the Breakdown of 4-methyl- α -hydroxyhippuric acid in H_2O , at 25°C , and $I = 1.0\text{M}$ (KCl).

Figure S8: Effect of Increasing Hydroxide Concentration of k_{obsd} on the Breakdown of α -hydroxyhippuric acid in H_2O , at 25°C , and $I = 1.0\text{M}$ (KCl).

Figure S9: Effect of Increasing Hydroxide Concentration of k_{obsd} on the Breakdown of 4-chloro-- α -hydroxyhippuric acid in H_2O , at 25°C , and $I = 1.0\text{M}$ (KCl).

Table S1: Kinetic Data of α -hydroxyhippuric acid

pH	ave k_{obsd}	log k_{obsd}
0	1.80E-6	-5.74
0.3	1.00E-6	-6.00
0.4	9.00E-7	-6.05
1	4.20E-7	-6.38
2.1	1.60E-7	-6.80
3.0	1.60E-7	-6.80
4.1	1.60E-7	-6.80
5.5	4.33E-7	-6.36
6.5	1.00E-6	-6.00
7.73	8.00E-6	-5.10
8.83	6.00E-5	-4.22
10.17	1.006E-3	-2.997
10.85	8.430E-3	-2.074
11.40	0.02090	-1.680
12.00	0.06966	-1.157
12.30	0.1191	-0.9240
12.48	0.1543	-0.8118
12.60	0.1890	-0.7235
12.70	0.2138	-0.6700
12.78	0.2302	-0.6379
12.85	0.2494	-0.6031
12.90	0.2752	-0.5604
12.95	0.2960	-0.5287
13.00	0.3146	-0.5022
13.30	0.4660	-0.3316
13.48	0.5753	-0.2401
13.60	0.6472	-0.1890
13.70	0.7122	-0.1474
13.70	0.7116	-0.1478
13.78	0.7598	-0.1193
13.85	0.7230	-0.1409
13.90	0.7834	-0.1060
13.95	0.8062	-0.09356
14.00	0.8522	-0.06946

Table S2: Kinetic Data of 4-Methyl- α -hydroxyhippuric acid

pH	ave k_{obsd}	log k_{obsd}
0	1.80E-6	-5.74
0.2	1.60E-6	-5.80
0.5	1.30E-6	-5.89
1	6.30E-7	-6.20
2.1	2.10E-7	-6.68
3.0	2.10E-7	-6.68
4.1	2.10E-7	-6.68
5.5	5.80E-7	-6.24
6.5	1.00E-6	-6.00
7.73	6.67E-6	-5.18
8.83	3.67E-5	-4.44
10.17	7.857E-4	-3.105
10.85	5.212E-3	-2.283
11.40	0.02476	-1.606
12.00	0.05244	-1.280
12.30	0.1220	-0.9136
12.48	0.1279	-0.8930
12.60	0.1550	-0.8097
12.70	0.1795	-0.7460
12.78	0.2012	-0.6964
12.85	0.2193	-0.6589
12.90	0.2366	-0.6260
12.95	0.2538	-0.5955
13.00	0.2688	-0.5706
13.30	0.3568	-0.4476
13.48	0.4464	-0.3503
13.60	0.5032	-0.2983
13.70	0.5548	-0.2559
13.70	0.5360	-0.2708
13.78	0.5666	-0.2467
13.85	0.6232	-0.2054
13.90	0.6324	-0.1990
13.95	0.6600	-0.1805
14.00	0.6900	-0.1611

Table S3: Kinetic Data of 4-(*N,N*-dimethylamino)- α -hydroxyhippuric acid

pH	ave k_{obsd}	log k_{obsd}
0	2.90E-6	-5.54
0.2	2.90E-6	-5.54
0.6	2.10E-6	-5.68
1	1.80E-6	-5.74
2.1	1.90E-7	-6.72
3.0	1.90E-7	-6.72
4.1	1.90E-7	-6.72
5.5	2.00E-7	-6.70
6.5	6.00E-7	-6.22
7.73	2.00E-6	-5.70
8.83	2.50E-5	-4.60
10.17	3.39E-4	-3.47
10.85	2.66E-3	-2.58
11.40	7.03E-3	-2.15
12.00	0.0278	-1.555
12.30	0.05886	-1.230
12.48	0.08354	-1.078
12.60	0.1052	-0.9780
12.70	0.1282	-0.8921
12.78	0.1466	-0.8339
12.85	0.1692	-0.7716
12.90	0.189	-0.7235
12.95	0.2082	-0.6815
13.00	0.2204	-0.6568
13.30	0.3360	-0.4737
13.48	0.4000	-0.3979
13.60	0.4840	-0.3152
13.70	0.5280	-0.2774
13.70	0.4852	-0.3141
13.78	0.5440	-0.2644
13.85	0.4860	-0.3134
13.90	0.5540	-0.2565
13.95	0.4600	-0.3372
14.00	0.5200	-0.2840

Table S4: Kinetic Data of 4-Methoxy-a-hydroxyhippuric acid

pH	ave k_{obsd}	log k_{obsd}
0	2.00E-6	-5.70
0.3	2.00E-6	-5.70
0.8	1.20E-6	-5.92
1	1.00E-6	-6.00
2.1	2.00E-7	-6.70
3.0	2.00E-7	-6.70
4.1	2.00E-7	-6.70
5.5	4.67E-7	-6.33
6.5	6.67E-7	-6.18
7.73	4.00E-6	-5.40
8.83	2.67E-5	-4.57
10.17	7.42E-4	-3.13
10.85	4.52E-3	-2.34
11.40	0.0122	-1.91
12.00	0.05504	-1.259
12.30	0.09980	-1.001
12.48	0.1336	-0.8742
12.60	0.1684	-0.7736
12.70	0.1934	-0.7135
12.78	0.2288	-0.6405
12.85	0.2416	-0.6169
12.90	0.2670	-0.5735
12.95	0.2714	-0.5664
13.00	0.3016	-0.5206
13.30	0.4200	-0.3768
13.48	0.4940	-0.3063
13.60	0.5260	-0.2790
13.70	0.5820	-0.2351
13.70	0.5414	-0.2666
13.78	0.5540	-0.2565
13.85	0.5650	-0.2480
13.90	0.5835	-0.2340
13.95	0.5520	-0.2581
14.00	0.5760	-0.2396

Table S5: Kinetic Data of 4-Chloro-a-hydroxyhippuric acid

pH	ave k_{obsd}	log k_{obsd}
0	8.20E-7	-6.09
0.1	7.00E-7	-6.15
0.6	4.30E-7	-6.37
1	1.10E-7	-6.96
2.1	1.00E-7	-7.00
3.0	1.00E-7	-7.00
4.1	1.00E-7	-7.00
5.5	6.00E-7	-6.22
6.5	3.00E-6	-5.52
7.73	1.67E-5	-4.78
8.83	2.00E-4	-3.70
10.17	2.02E-3	-2.70
10.85	0.0112	-1.95
11.40	0.0300	-1.52
12.00	0.1139	-0.9434
12.30	0.1816	-0.7409
12.48	0.2226	-0.6525
12.60	0.2700	-0.5686
12.70	0.3060	-0.5143
12.78	0.3400	-0.4685
12.85	0.3794	-0.4209
12.90	0.4016	-0.3962
12.95	0.4318	-0.3647
13.00	0.4640	-0.3335
13.30	0.6886	-0.1620
13.48	0.8300	-0.08092
13.60	0.9580	-0.01863
13.70	1.036	0.01536
13.70	1.034	0.01452
13.78	1.024	0.01030
13.85	1.060	0.02531
13.90	1.172	0.06893
13.95	1.240	0.09342
14.00	1.278	0.1065

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