Identification and Characterization of Atrazine-Degrading Phytochemicals in Eastern Gamagrass

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by

CAMMY DROST WILLETT

Drs. Robert N. Lerch and Keith W. Goyne, Dissertation Supervisors

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The undersigned, appointed by the Dean of the Graduate School,

have examined the dissertation entitled

Identification and Characterization of Atrazine-Degrading

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Presented by Cammy Drost Willett

A candidate for the degree of

Doctor of Philosophy

And hereby certify that, in their opinion, it is worthy of acceptance.

Robert N. Lerch (Chair)

Keith W. Goyne (Co-Chair)

Chung-Ho Lin

Craig A. Roberts

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ABSTRACT

Naturally occurring phytochemicals of the 1,4-benzoxazin-3-one (Bx) class exhibit broad chemical reactivity that includes detoxification of chloro-triazine herbicides. Atrazine (6-chloro-*N*-ethyl-*N'*-(1-methylethyl)-1,3,5-triazine-2,4-diamine) contamination of surface and ground water remains an environmental concern that warrants continued research to identify mitigation and decontamination strategies. In this research, the Bx compound DIBOA-Glc (2-β-D-glucopyranosyloxy-4-hydroxy-1,4-benzoxazin-3-one) was identified as the atrazine-degrading compound present in eastern gamagrass roots (EG, *Tripsacum dactyloides* (L.) L.); identifying Bx compounds in the *Tripsacum* species for the first time. In order to further characterize the reaction between atrazine and DIBOA-Glc, methodology was developed in which bulk quantities of highly pure DIBOA-Glc were produced using HPLC separation of crude methanol extracts of EG roots. The resulting pure DIBOA-Glc was then used to characterize the atrazine-DIBOA-Glc reaction in terms of reaction kinetics, identification of intermediates and products, and pH effects. Findings from these studies demonstrated that DIBOA-Glc was consumed in the reaction and was not acting catalytically. A conjugate of DIBOA-Glc and atrazine was identified as a stable reaction intermediate. Additionally, the formation of hydroxyatrazine (2-hydroxy-4-ethylamino-6-isopropylamino-*s*-trazine) and Cl^- as reaction products confirmed that the reaction mechanism was a nucleophilic attack of the hydroxamic acid moiety at the C-2 position. Use of naturally occurring phytochemicals, such as DIBOA-Glc, in concert with existing mitigation practices may allow producers to continue the use of atrazine as a valuable crop protection tool while simultaneously protecting the soil and water resources in environments where atrazine is used.

CHAPTER 1: OVERVIEW

Introduction

Naturally occurring phytochemicals have long been sought after for various beneficial properties. Compounds derived from plants with medicinal, cultural, religious, and agronomic applications have fascinated and frustrated researchers since the beginning of scientific inquiry (Mclaughlin 1973, Dias et al. 2012). One such class of compounds, benzoxazinoids (Bx), have been studied by many disciplines over the last six decades.

Benzoxazinoids have the generic structure 2-hydroxy-2*H*-1,4-benzoxazin-3(4H)-one, and are known to possess a wide range of potentially beneficial properties including anti-tumor and anti-cancer (Zhang et al. 1995, Roberts et al. 1998, Joshi et al. 1999), anti-inflammatory (Otsuka et al. 1988), anti-fungal, anti-microbial, insecticidal (Niemeyer et al. 1992), and herbicidal characteristics (Niemeyer 1988, Sicker and Schulz 2002, Niemeyer 2009). They are involved in metal chelation and metabolism in plants (Tipton and Buell 1970), can influence reproduction in rodents (Berger et al. 1981), and participate in a wide range of allelopathic processes (Sicker and Schulz 2002, Rice et al. 2005, Schulz et al. 2013). In addition, Bx compounds are capable of detoxifying xenobiotics, including triazine compounds such as the corn herbicide atrazine (6-chloro-*N*-ethyl-*N*′-(1-methylethyl)-1,3,5-triazine-2,4-diamine) (Castelfranco and Brown 1962, Raveton et al. 1997, Willett et al. 2013). It is at the intersection of natural products

chemistry and environmental quality that the research presented in this dissertation originates.

Long-term and widespread use of atrazine in corn production has led to significant levels of the herbicide in surface and ground water supplies (Pereira et al. 1990, Squillace and Thurman 1992, Koplin et al. 1998, Lerch et al. 1998, Battaglin et al. 2003, Lerch and Blanchard 2003). However, as an effective and economically viable herbicide, producers across the Midwest continue to rely on it as a valuable crop protection tool. The degradation pathways of atrazine in plants (Davis et al. 1965, Jensen et al. 1977, Burken and Schnoor 1997, Cherifi et al. 2001), soil (Armstrong et al. 1967, Gamble and Khan 1985), and water (Lerch et al. 1998), and mitigating atrazine contamination of surface and ground water in agricultural watersheds have been extensively studied (Gaynor et al. 1995, Barbash et al. 2001, Reichenberger et al. 2007, Lerch et al. 2008, Lerch et al. 2011). Environmental stewardship and increased sustainability of crop production systems urges continued research on atrazine mitigation and decontamination strategies.

Phytochemical Degradation of Atrazine

The biological degradation pathways of atrazine in tolerant plant species, such as corn (*Zea mays* L.), have been comprehensively studied (Shimabukuro et al. 1970, Lamoureux et al. 1973, Ohkawa et al. 1999). Phytoremediation studies have focused on metabolic pathways that atrazine resistant plants employ to degrade the herbicide and application of such findings for mitigating atrazine contamination of soil and water. In atrazine resistant plants, the herbicide is metabolically degraded into nontoxic metabolites by hydroxylation, N-dealkylation, and conjugation to glutathione (Jensen et

al. 1977, Raveton et al. 1997, Cherifi et al. 2001). Through decades of research, three biological degradation pathways have emerged, including two enzymatic pathways and one chemical pathway. The naturally occurring enzymes present in atrazine resistant plants include glutathione transferase (GST) (Jensen et al. 1977, Cole and Edwards 2000, Andrews et al. 2005, Marcacci et al. 2006) and cytochrome P450 (Kawahigashi et al. 2006). Non-enzymatic chemical degradation is mediated by Bx compounds, such as DIMBOA (2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one) and DIBOA (2,4-dihydroxy-2H-1,4-benzoxazin-3(4H)-one) and their glucosides (Castelfranco et al. 1961, Raveton et al. 1997, Merini et al. 2009, Willett et al. 2013). Enzymatic and non-enzymatic atrazine tolerance mechanisms have been described in a wide array of plants (Marcacci et al. 2006, Merini et al. 2009).

Glutathione conjugation is a common detoxification pathway for many xenobiotics, including herbicides (Jensen et al. 1977, Hoagland et al. 2000, Andrews et al. 2005). Glutathione S-transferases are a group of enzymes found in all eukaryotes that catalyze the conjugation of synthetic electrophilic compounds with the tripeptide glutathione (Andrews et al. 2005). Atrazine conjugation to glutathione as a detoxification route in corn was first described by Shimaburkuro et al. (1971). Before conjugation, xenobiotics are initially hydrolyzed or oxidized and then subjected to glutathione conjugation (Hoagland et al. 2000). Atrazine metabolism by glutathione conjugation occurs through nucleophilic attack by the thiolate anion of glutathione with carbon at the C-2 position of the triazine ring which has an ion induced dipole associated with covalent bonding to chlorine (Ohkawa et al. 1999, Cole and Edwards 2000). Conjugate formation detoxifies atrazine (Ohkawa et al. 1999), followed by conjugate processing through a

complex pathway that is initiated by stepwise peptide cleavage. The fate of these conjugates in sorghum is described in detail by Lamoureux et al. (1973).

Cytochrome P450 detoxification is an additional enzymatic pathway important for degrading and eliminating xenobiotics, such as atrazine, within plants (Coleman et al. 1997, Harvey et al. 2002, Merini et al. 2009). There are two cytochrome P450 monooxygenases-- cytochrome P450 and NADPH-cytochrome P450 oxidoreductase-- that catalyze atrazine degradation through oxidation (Ohkawa et al. 1999, Hoagland et al. 2000). Cytochrome P450 is thought to mediate N-dealkylation (Ohkawa et al. 1999, Marcacci et al. 2006) of either side chain (Jensen et al. 1977), although this has not been proven *in vitro* (Ohkawa et al. 1999). The products of these reactions can then be further processed and detoxified by conjugation with molecules such as glucose and other water soluble moieties (Hatzios 1997, Ohkawa et al. 1999).

The reaction of the triazine herbicide simazine (6-chloro-*N,N'*-diethyl-1,3,5-traizine-2,3-diamine) and Bx compounds extracted from corn was first established by Castelfranco et al. (1961, 1962), and a reaction mechanism was proposed. Raveton et al. (1997) found that 91% of atrazine, reacted with a crude acetone extract of Bx compounds, degraded to hydroxyatrazine (HA; 2-hydroxy-4-ethylamino-6-isopropylamino-*s*-triazine) following a 24 hour incubation at 25°C. The optimal pH for the first order reaction was pH 5.5, and the half-life of atrazine decreased with increasing temperature (Raveton et al., 1997).

Benzoxazinoid mediated degradation of triazines is the only phytochemically-mediated degradation reaction presently documented in the literature for the triazine class of herbicides. While a handful of mechanisms have been proposed to describe the

reaction in detail, none have been substantiated. One such proposal, put forth by Raveton et al. (1997), hypothesized the formation of HA and a stable chlorinated Bx product

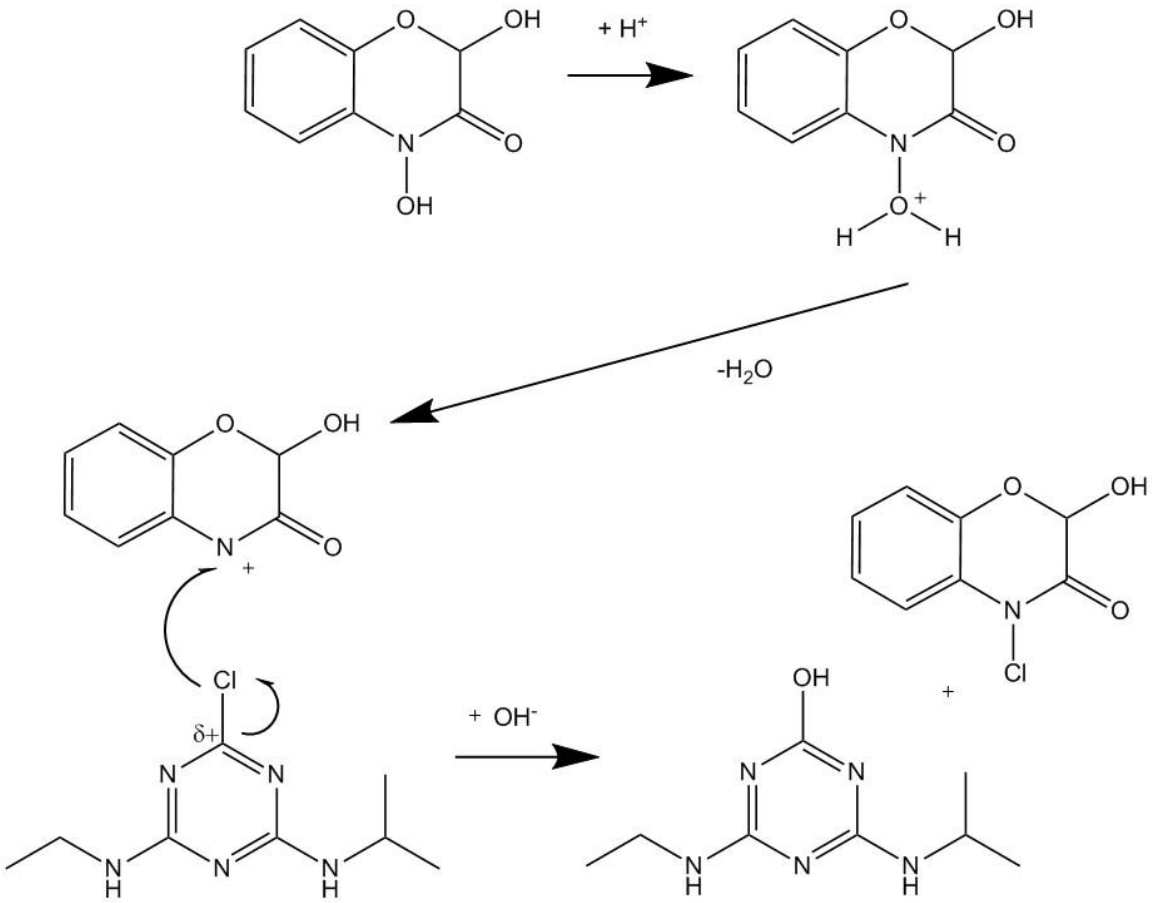

Figure.1.1 Hypothetical mechanism for atrazine hydroxylation by benzoxazinones where the Bx compound is protonated previous to reacting with atrazine (adapted from Raveton et al. 1997).

(Figure 1.1). The formation of an electrophilic N^+ intermediate by spontaneous loss of water and subsequent chlorination seem unlikely given the presence of the nucleophilic hydroxamic acid and electrophilic C-2 of the triazine ring. Regardless, the chlorinated Bx product should be readily detected by current LC/MS/MS instrumentation, yet no studies to date have confirmed the presence of this product. Thus, the need exists for identification of the Bx products formed by this reaction. Others have used model compounds that were more stable or easier to obtain in high purity to study the Bx-

triazine reaction, such as *N*-hydroxysuccinimide, 1-hydroxy-2-piperidone (Nakano et al. 1973), pyridine and hydroxylamine (Castelfranco and Brown 1962). From these and other studies, the presumed mechanism involving Bx phytochemicals and triazines is a nucleophilic attack at the C-2 position of the triazine ring (Figure 1.2) (Castelfranco et al. 1961, 1962, Nakano et al. 1973). While this is a probable reaction mechanism and the formation of HA as the atrazine product is well established, use of model compounds to study nucleophilic attack of atrazine cannot elucidate the formation of Bx degradation products. Additionally, there is debate among the literature on the final fate of the Bx compound and whether or not it reacts catalytically with the triazine or is consumed in

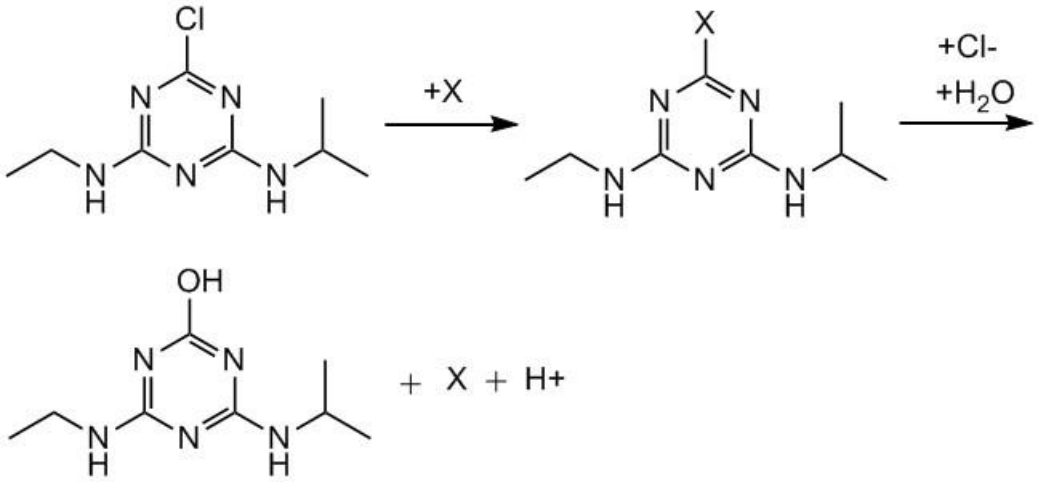

Figure 1.2. Hypothetical mechanism for Bx-catalyzed atrazine hydroxylation by corn root extracts (adapted from Castelfranco et al. 1961).

the reaction (Hamilton and Moreland 1962, Tipton et al. 1971, Nakano et al. 1973, Niemeyer 1988, Raveton et al. 1997, Niemeyer 2009).

Occurrence of Benzoxaxinoids Compounds in Plants

Several members of the Panicoideae subfamily (e.g., corn, prosomillett (*Panicum miliaceum* L.), and large crabgrass (*Digitaria sanguinalis* (L.) Scop.) have high atrazine metabolism rates (Jensen et al. 1977), and understanding metabolism is important for

pesticide bioremediation in contaminated soil and water (Hoagland et al. 2000). The C_4 cycle of panicoid species results in rapid CO_2 assimilation rates and a lack of photorespiration and helps impart tolerance to atrazine (Jensen et al. 1977). In additional to these characteristics, several agronomically important crops such as corn, wheat (*Triticum* L.), and rye (*Secale* L.) produce Bx compounds. Due to their agronomic importance, occurrence of Bx compounds in these species has been extensively studied (Zuniga et al. 1983, Niemeyer et al. 1992, Gianoli and Niemeyer 1998, Sicker and Schulz 2002, Rice et al. 2005, Pedersen et al. 2011).

Forage grasses have been identified as viable phytoremediation agents for herbicides and other organic compounds (Lin et al. 2008, Yang 2010). Eastern gamagrass (*Tripsacum dactyloides* (L.) L.; EG) is also in the panicoideae subfamily, and is a warm-season grass native to Missouri and a species often grown and used for forage. Along with the characteristics of a C_4 plant, EG is known to be flood tolerant (Euliss et al. 2008) and promotes degradation of xenobiotics such as petroleum products (Euliss et al. 2008) and the triazine explosive 1,3,5-trinitrohexahydro-s-triazine (RDX) (Yang 2010). Given these characteristics, it is a strong candidate for phytoremediation of triazines in contaminated soil and water.

While grasses have shown potential for point-source clean-up of pesticides (Belden et al. 2003), incorporation of grasses into vegetative buffer strips has similar potential to mitigate non-point source herbicide contamination of surface and ground water (Lin et al. 2003, 2011). However, vegetative buffer strips or stands of grass alone are not capable of achieving the needed reductions in herbicide transport. Thus, a systematic assessment of phytochemicals in atrazine tolerant forage species, such as EG,

will likely lead to the discovery of herbicide degrading chemicals and could lead to the development of a chemical product for use with preexisting phytoremediation practices to further enhance degradation and eliminate atrazine contamination of soil and water resources.

Study Objectives

The overall objective and unifying theme of this research was to identify and characterize atrazine-degrading compounds isolated from EG. To meet this objective, several studies were conducted:

Study 1- The specific objective of Study 1 was to identify atrazine-degrading compounds in EG roots. Standard techniques such as solvent extraction, liquid/liquid extraction, and HPLC fraction-collection were used to isolate active phytochemicals. Material isolated during the separation processes were tested for atrazine-degrading activity using a simple assay. An iterative process of fractionation and testing for atrazine-degradation activity was used until compound identification could be achieved. Full details of this study were published in Willett et al. (2013) and comprise the contents of Chapter 2.

Study 2- The specific objective of Study 2 was to isolate and purify the compound identified in Study 1 for use in other studies, as the phytochemical of interest is not currently available for purchase. Methods available in the literature had various disadvantages such as a need for expertise as a synthetic chemist, access to equipment not widely available, use of hazardous chemicals, or a lack of definitive compound identification and purity information of the material produced. Methods were developed to isolate and purify the previously identified Bx compound using aqueous methanol root

extracts and HPLC fraction separation. The purity of the isolated material was quantified and found suitable for use as a quantitative standard for subsequent studies. A manuscript of the method has been published (Willett et al. 2014) and is included in Chapter 3.

Study 3- Characterization of the atrazine/phytochemical degradation reaction was the objective of this series of experiments. The reaction was characterized in terms of pH effects, kinetics, mechanism, and stoichiometry. This was accomplished by manipulating reaction solution pH, forcing pseudo-first order kinetics with excess atrazine or phytochemical to isolate degradation kinetics of each reactant, and by using chromatographic and spectroscopic techniques to identify and quantify reaction products to infer reaction mechanism. While atrazine products of the atrazine/phytochemical reaction are well documented, a main goal of the current work was to provide information regarding the fate of the phytochemical after the degradation reaction via identification of Bx reaction products. Chapter 4 contains the details of the Study 3 experiments.

Conclusion

Data obtained from all phases of research brings unity and clarification to the project findings, ultimately leading to an assessment of the characterized compound for use in atrazine remediation and mitigation. Use of naturally occurring phytochemicals in concert with existing mitigation practices may allow producers to continue the use of atrazine as a valuable crop protection tool while simultaneously protecting the soil and water resources in environments where atrazine is used. While these investigations stem from an interest in using Bx compounds to mitigate environmental contamination of atrazine, it makes a broader contribution to the long and multifaceted history of scientific

interest surrounding these remarkable compounds. The impact and utility of this research could inform research in a diverse range of fields and help advance natural products research.

Adapted with permission from Willett, C. D., R. N. Lerch, C. H. Lin, K. W. Goyne, N. D. Leigh and C. A. Roberts (2013). Identification of an atrazine-degrading benzoxazinoid in Eastern gamagrass (Tripsacum dactyloides). *J Agric Food Chem* **61**(34): 8026-8033.

CHAPTER 2: IDENTIFICATION OF AN ATRAZINE-DEGRADING BENZOXAZINOID IN EASTERN GAMAGRASS (*TRIPSACUM DACTYLOIDES*)

Abstract

This study was part of a broader effort to identify and characterize promising atrazine-degrading phytochemicals in Eastern gamagrass (*Tripsacum dactyloides*; EG) roots for the purpose of mitigating atrazine transport from agroecosystems. The objective of this study was to isolate and identify atrazine-degrading compounds in EG root extracts. Eastern gamagrass roots were extracted with methanol and extracts were subjected to a variety of separation techniques. Fractions from each level of separation were tested for atrazine-degrading activity by a simple assay. Compounds were identified using high performance liquid chromatography-tandem mass spectrometry. Results from the experiments identified 2-β-D-glucopyranosyloxy-4-hydroxy-1,4-benzoxazin-3-one (DIBOA-Glc) as the compound responsible for atrazine degradation in the root extract fractions collected. 2-β-D-glucopyranosyloxy-1,4-benzoxazin-3-one (HBOA-Glc) was also identified in the root extract fractions, but it did not demonstrate activity against atrazine. Estimated root tissue concentrations were 210 mg kg^{-1} (wet wt. basis) for DIBOA-Glc and 71 mg kg^{-1} for HBOA-Glc (dry wt. basis 710±96 mg kg^{-1} and 240±74

mg kg^{-1}, respectively). This research was the first to describe the occurrence and concentrations of an atrazine-degrading benzoxazinone compound isolated from EG tissue.

Introduction

Atrazine (1-Chloro-3-ethylamino-5-isopropylamino-2,4,6-triazine) contamination of water resources continues to be a problem across the Midwestern United States (Squillace and Thurman 1992, Thurman et al. 1992, Lerch et al. 1995, Fallon et al. 2002, Battaglin et al. 2003, Lerch et al. 2011). Research has aided in the development of agricultural practices for mitigating and preventing atrazine contamination of water resources, such as herbicide incorporation (Gaynor et al. 1995, Ghidey et al. 2010), reduced application rates, and establishment of vegetative buffers (Lin et al. 2004, 2008, Lin et al. 2011). Despite intensive efforts to mitigate atrazine transport over the last several decades, research scientists, policy makers, farmers and the general public are still concerned with the threats posed by the presence of atrazine in surface and ground water supplies (Fallon et al. 2002, Brands and Rajagopal 2008).

Benzoxazinoid (1,4-benzoxazin-3-one; Bx) compounds, common in many species of the Poaceae family, including several agronomically important crops such as corn (*Zea mays*), wheat (*Triticum aestivum*), and rye (*Secale cereal*) have been extensively studied due to the wide range of potential benefits they can provide (Niemeyer 1988, Marcacci et al. 2006, Frey et al. 2009, Rice et al. 2012). These compounds are known to be allelopathic and impart resistance to fungi, bacteria, and insects (Niemeyer 1988, Niemeyer et al. 1992, Gianoli and Niemeyer 1998, Fomsgaard et al. 2004, Niemeyer 2009). Due to the allelopathic nature of these compounds, use of these biomolecules as

natural herbicides has been investigated (Macias et al. 2005). Interestingly, it has also long been known that Bx compounds impart resistance to triazine herbicides such as atrazine and simazine (Castelfranco et al. 1961, Castelfranco and Brown 1962). However, their potential utility for mitigating atrazine transport from cropland and remediating atrazine contamination of water resources has not been investigated.

Findings from previous work involving the screening of native grass species for use in vegetative buffers suggested the presence of one or more compounds that enhance atrazine degradation in the rhizosphere of warm season grasses (Lin et al. 2008, Lin et al. 2011). Lin et al. (2011) showed that rhizosphere soil cultivated with the warm season species, Eastern gamagrass (*Tripsacum dactyloides* L.; EG), degraded 90% and mineralized nearly 7% of the added atrazine during a 115 d incubation trial, the most of the seven grass and forage species tested. In addition, Lin et al. (2011) reported that atrazine exposed to EG root extracts was rapidly hydrolyzed to the less toxic metabolite hydroxyatrazine. They speculated that the presence of Bx compounds in the rhizosphere soil of warm season grasses may have contributed to the enhanced atrazine degradation compared to cool season species. However, the identification of these possible atrazine-degrading phytochemicals was beyond the scope of the studies by Lin and colleagues (Lin et al. 2008, Lin et al. 2011). Yang (2010) also reported that the degradation of the triazine explosive 1,3,5-trinitroperhydro-1,3,5-triazine (RDX) was enhanced in the rhizosphere soil of EG plants.

Based on these previous studies, a preliminary experiment was performed to confirm the ability of EG root extracts to degrade atrazine. A simple assay showed positive results; therefore, a general extraction and isolation scheme was developed and

initiated for the study presented here. Although Bx compounds have been shown to impart atrazine resistance in several species of Poaceae (Niemeyer 1988, Marcacci et al. 2006, Frey et al. 2009), the present study employed a general screening process for the presence of novel phytochemicals as well as Bx compounds. Thus, the methods were intentionally developed as a broad approach for targeting compounds of interest and not an optimized method for Bx compound isolation and quantification. However, based on reports of Bx occurrence across species, the presence of Bx compounds in *Tripsacum* species has apparently been considered common knowledge among researchers in the field since the late 1980's (Niemeyer 1988, Marcacci et al. 2006, Frey et al. 2009). Further investigation of the cited sources revealed that claims of Bx compound occurrence in *Tripsacum* were not substantiated in the peer-reviewed literature. An important purpose of the current research was to provide peer-reviewed evidence to substantiate or refute the claim that Bx compounds occur in *Tripsacum*.

The broader aim for the study was to identify and characterize the most promising atrazine-degrading compounds in EG for the purpose of developing a new approach for mitigating atrazine contamination of water resources. We envision that the use of an atrazine-degrading phytochemical in concert with other practices proven to reduce atrazine in surface runoff could improve water quality. For example phytochemical incorporation into an existing vegetative buffer could be used to further enhance the degradation of atrazine and minimize atrazine loss from agroecosystems. The specific objective for the research presented here was to isolate and identify atrazine-degrading compounds found in EG root extracts.

Materials and Methods

Chemicals. Chromatography grade solvents and reagents including acetonitrile (CH_3CN), chloroform, dichloromethane, ethyl acetate, methanol (CH_3OH), and concentrated phosphoric acid (H_3PO_4) were purchased from Fisher Scientific. Atrazine (98.8% purity) was obtained from ChemService, Inc. (West Chester, PA). Radio-labeled ^{14}C-atrazine was purchased from America Radiolabeled Chemicals, Inc. (St. Louis, MO). Ultrapure Millipore water was used for sample preparation and analysis unless otherwise noted.

Root Collection. Samples of EG plants were collected from the University of Missouri Bradford Research and Extension Center, near Columbia, Missouri. Plants were collected from an EG plot established before 2000 that had not been treated with atrazine for at least 10 years. Whole plants, including roots and leaves, were excavated. Plants were washed with tap water on site to remove loose soil. In the laboratory, the leaves and stems were separated from the roots. Roots were rinsed with tap water followed by reverse osmosis water to remove as much remaining soil as possible. The roots were stored in polyethylene bags at -4ºC and the leaves and stems were discarded. Sample preparation procedures following root collection are outlined in Figure 2.1.

Root Extracts. A preliminary experiment was performed to determine if CH_3OH or ethyl acetate produced an extract with the greatest amount of reactivity against atrazine. Root samples of 50 g wet weight were homogenized in a Waring blender with 100 mL of 9:1 CH_3OH:H_2O (v:v) or ethyl acetate. Triplicate samples of each solvent were prepared, transferred to polypropylene bottles, and shaken end-to-end overnight. After shaking, the homogenate samples were vacuum filtered through Whatman 42 ashless filters to remove root solids. The filtrate was transferred to a collection beaker and the filter paper

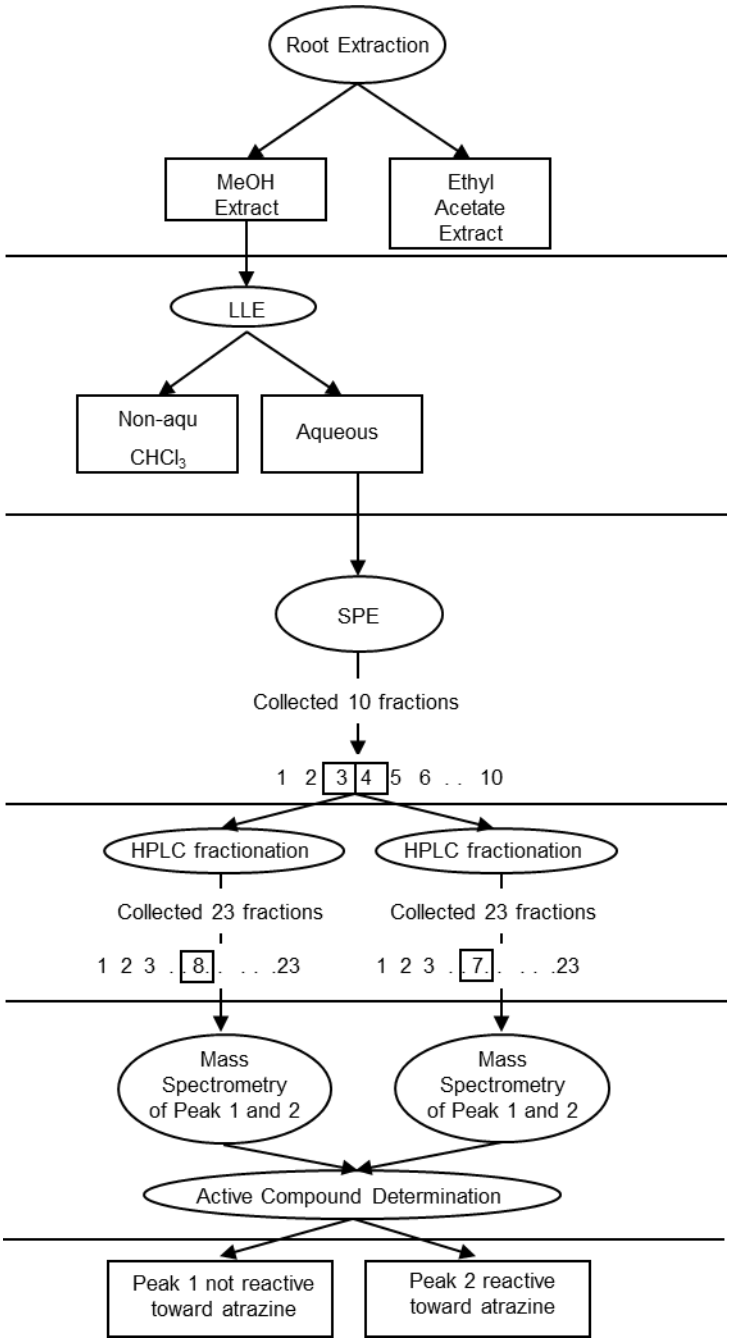

Figure 2.1 Schematic detailing the extraction, separation, and identification of atrazine-degrading phytochemicals from Eastern gamagrass (*Tripsacum dactyloides*). Corresponding materials and methods sections appear in the oval shapes. Horizontal lines between sections represent application of an atrazine assay and analysis of fractions for atrazine degradation.

discarded. The homogenate was rinsed with an additional 50 mL of solvent (CH_3OH or ethyl acetate), filtered with a new filter paper, and combined with the first filtrate in the collection beaker. The combined filtrate was concentrated at 35 to 40°C with a stream of N_2 to ~10 mL. A 500 µL aliquot was removed from each sample and evaporated to dryness and re-suspended in 1 mL water.

Atrazine Assay and HPLC Analysis. Samples resulting from each level of separation and fractionation as outlined in Figure 2.1 were subjected to a simple atrazine assay to determine the fraction's ability to degrade the herbicide. The assay entailed spiking each sample with atrazine in aqueous solution, allowing the solution to react at ambient laboratory temperature (22-25°C) overnight, and analyzing for the remaining atrazine. Each assay also included water controls (containing only atrazine) which were treated in the same manner as the samples for the assay and analysis. Details of the assay parameters for each level of separation are provided in the following sections. The results of the atrazine assays varied considerably depending upon the stage of fractionation (Figure 2.1). Concentrations of Bx compounds in plant tissue have been shown to be highly variable and depend upon growth stage, type of tissue, and length and density of roots (Cambier et al. 1999, Baumeler et al. 2000). These factors partially explained the variation between the degradation of early fractions and the later DIBOA-Glc and HBOA-Glc fractions as root age, growth stage, and storage time of the extracted material were not controlled for in these experiments. In addition, procedural details at each step (see below) varied and resulted in dilution or loss of compound mass through the fractionation processes. Final concentrations of active compounds in the fractions could not be controlled for as their identity was unknown in all but the last set of fractions.

For the root extracts described above, the 1 mL samples were spiked with 200 µL of a 10 mg L^{-1} atrazine solution in 1:9 CH_3OH:H_2O (v:v, 10% CH_3OH hereafter) to achieve an initial atrazine concentration of 1.667 mg L^{-1} in the reaction vessel. Reaction vessels were covered in aluminum foil and reacted on the benchtop for ~16 hours. Atrazine was extracted from the sample three times via liquid/liquid extraction (LLE) using a total of 15 mL of chloroform. The organic phase containing atrazine was collected with pasture pipettes and evaporated to dryness. Samples were re-suspended in 1.2 mL of water and analyzed using reverse-phase high performance liquid chromatography (HPLC) as described by Lin et al. (2011). In brief, a Shimadzu LC-10AT VP (Columbia, MD) HPLC equipped with a UV detector was used for analysis. The column was a Columbus C8 (250x4.60 mm, 5 µm; Phenomenex, Torrance, CA), and separation was achieved with a 0.1% H_3PO_4 /CH_3CN gradient mobile phase at 1 mL min^{-1} flow rate. UV detection was at 254 nm, and a 25 µL injection volume was used. The concentration of atrazine remaining for the two solvent treatments and water controls were compared using a one-way analysis of variance (ANOVA), α=0.05. Significant differences between treatment means were determined using Tukey's HSD.

Liquid/Liquid Extraction Fractionation. Samples for liquid/liquid extraction (LLE) fractionation were prepared in a similar manner as described in the root extracts section. Root samples (100 g wet weight) were blended with 200 mL of 9:1 CH_3OH:H_2O, and 100 mL CH_3OH was used for the second extraction. To remove the CH_3OH, the filtrates were evaporated to a final volume of 15 to 20 mL. Concentrated aqueous samples were sonicated for 5 to 10 min and quantitatively transferred to a separatory funnel with 100 mL (50 mL x 2) of chloroform or dichloromethane for LLE. The organic phase was

collected and the aqueous phase extracted with an additional 100 mL of chloroform or dichloromethane. The emulsion layer was collected and centrifuged to complete the phase separation. The resulting organic and aqueous fractions were then evaporated to ~5 mL.

For the assay, aliquots of 250 μL were removed from the concentrated aqueous and organic fractions and evaporated to dryness. The samples were spiked with 200 μL of 2.5 mg L^{-1} atrazine solution in 10% CH_3OH. This quantity included 0.125 μCi mL^{-1} of ^{14}C-labeled atrazine. Water was added to each sample to bring the final volume to 1.1 mL, thereby achieving a 1.11 mg L^{-1} initial concentration. In early experiments, solvent was added at the end of the reaction period in an attempt to stop the reaction. Later evidence indicated that liquid/liquid extraction was a more appropriate procedure for stopping the reaction. For assays where solvent was added at the end of the reaction period, a 500 μL aliquot of CH_3OH was added and samples were placed in cold storage (4°C) until analysis. The assays were performed in duplicate. Samples and water controls were analyzed for atrazine using HPLC-UV and in-line flow scintillation analyzers as described in Lin et al. (2011), using an injection volume of 25 μL.

Peak area results of the atrazine assay for the aqueous and organic treatments and water controls were compared using a one-way ANOVA, α=0.05. Significant differences between treatment means were determined using Tukey's HSD.

Solid-Phase Extraction Fractionation. Solid-phase extraction (SPE) cartridges (Applied Separations, Allentown, PA) packed with RP-102 (styrene-divinylbenzene resin; 2.5 g 20 mL^{-1}) were used to further purify the aqueous fraction samples derived from LLE of the CH_3OH root extracts. SPE cartridges were conditioned with 40 mL of CH_3CN followed

by 50 mL of water. Using gravity-flow, 3 mL of aqueous sample was loaded evenly on the cartridge. The SPE cartridges were eluted with 200 mL of 1:9 $CH_3CN:H_2O$ and collected into separate fractions (referred to as SPE fractions hereafter). Using an ISCO Foxy 200 fraction collector (Lincoln, NE), 30 SPE fractions of 170 drops each were collected from each cartridge. Preliminary analysis (data not shown) of the 30 SPE fractions showed that only the first ten fractions exhibited reasonable activity against atrazine (i.e., >40% degraded). Based on this information, only the first ten SPE fractions were collected. The collection procedure was replicated 10 times. Volumes of the SPE fractions were 6 to7 mL and were concentrated to a final volume of 2 mL.

For the assay, a 150 μL aliquot from each fraction was reacted with 30 μL of 10 mg L^{-1} atrazine in 10% CH_3OH under conditions as previously described. Thirty microliters of CH_3CN were added at the end of the reaction period and samples were held in cold storage until analysis by HPLC-UV as described in the atrazine assay and HPLC analysis section.

HPLC Fractionation. The SPE fractions that exhibited the ability to degrade at least 50% of the atrazine during the atrazine assay (3rd through 8th SPE fractions; Figure 2.1) were further fractionated using HPLC (referred to as HPLC fractions hereafter). The HPLC conditions were similar to those used for the atrazine assay analysis (Lin et al. 2011). The mobile phase was modified to $CH_3CN:H_2O$ and a 100 μL injection volume was used. A Shimadzu FRC-10A fraction collector was employed to collect 23 fractions of 2 mL each from the selected SPE fractions over the first 46 min of the 60 min method; the remaining time was used for column flush and equilibration. The result was 23 HPLC fractions from each of the 3rd through 8th SPE fractions injected. Duplicates were collected from each

SPE fraction. The 2 mL samples were evaporated to dryness and re-suspended in 150 μL water and spiked with atrazine to an initial concentration of 1.667 mg L^{-1}. An atrazine assay followed by HPLC-UV was carried out as described above.

Analysis of the 23 HPLC fractions showed that two fractions from SPE fractions 3 and 4 degraded atrazine to the greatest extent. The HPLC fractionation was then repeated 50 times using these two SPE fractions for injection material (Figure 2.1). The 50 samples collected from each SPE fraction were combined and evaporated to 5 mL. The atrazine-degrading fractions were collected in a 2-minute window from 9.8 to 11.8 min that contained two distinct peaks (Peak 1 and Peak 2). The ability of these samples to degrade atrazine was confirmed by the atrazine assay and HPLC-UV analysis. The HPLC fractions collected from SPE fractions 3 and 4 (Figure 2.2) were later shown to contain the same compounds; thus, they were combined for subsequent processing steps.

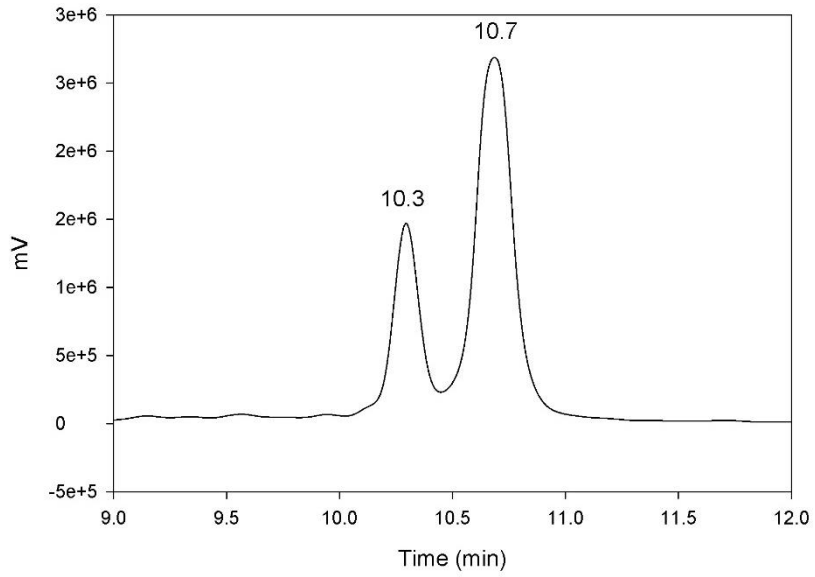

Figure 2.2 Chromatograph (UV λ=254 nm) demonstrating Peak 1 and Peak 2 in atrazine-degrading fractions derived from HPLC fractionation of SPE fractions 3 and 4. Retention times were Peak 1= 10.3 min; Peak 2= 10.7 min.

Mass Spectrometry. Tentative identification of Peak 1 and Peak 2 (Figure 2.2) was obtained by high performance liquid chromatography-tandem mass spectrometry with simultaneous diode array detection (LC/MS/MS-DAD). Analyses were carried out using a Thermo-Finnigan TSQ7000 triple-quadrupole mass spectrometer, equipped with an API2 source and Performance Pack (Thermo Electron Corp., San Jose, CA). Samples were introduced into the TSQ7000 via an integrated Thermo-Finnigan LC system consisting of a P4000 quaternary LC pump and a UV6000LP diode-array detector. The LC method from Lin et al. (2011) was used. Electrospray ionization and atmospheric pressure chemical ionization, positive and negative modes, were investigated, and atmospheric pressure chemical ionization in positive ion mode gave the best ionization. The heated inlet capillary was maintained at 250ºC, the vaporizer temperature was 400ºC, and the corona discharge current was 5.00 μA. Other voltages were optimized during regular tuning and calibration to maximize ion transmission and minimize unwanted fragmentation. The sheath gas (80 psi) and auxiliary gas (40 arbitrary units on the integrated flowmeter) was N_2. Argon was used as the collision gas for MS/MS experiments. By comparing the pseudomolecular ion ($[M+H]^+$) masses and fragmentation patterns of the precursor ion spectra to that of known Bx compounds in the literature (Cambier et al. 1999), the peaks were tentatively identified. The tentative identifications were further verified against authentic standards. HBOA-Glc (2-β-D-glucopyranosyloxy-1,4-benzoxazin-3-one) was purified using methods from Yin et al. (2008), DIBOA-Glc (2-β-D-glucopyranosyloxy-4-hydroxy-1,4-benzoxazin-3-one) was purified using methods from Kluge et al. (1997), as cited in Rice et al. (2005), and were provided by Dr. Clifford Rice of USDA-ARS, Beltsville, MD. The authentic standards were useful for compound

identification, but their concentrations were only approximately known because they had been in cold storage for several years prior to acquisition and therefore could not be used for quantifying isolated Bx compound concentrations in EG.

Estimation of Bx concentrations in EG root tissue was performed by LC/MS/MS-DAD at 255 nm. A molar absorptivity coefficient of 8,500 was used to estimate the concentration of each compound in sample solutions based on the Beer-Lambert Law as standards of known concentration were not available (Bailey and Larson 1989). Four CH_3OH root extracts were prepared as described in the root extract section, except that the root and solvent quantities were doubled, and samples were concentrated to ~15 to 20 mL. Each sample was analyzed for absorption at 255 nm with LC-DAD.

Active Compound Determination. Mass spectral analysis confirmed that the peaks in the active fractions derived from SPE fractions 3 and 4 were identical, and so the samples were combined (Figure 2.1). The peaks were designated as Peak 1 and Peak 2 based on their retention times - 10.3 min for Peak 1 and 10.7 min for Peak 2 (Figure 2.2). To separate the peaks into distinct fractions, the sample was separated by HPLC. Fraction collection was performed based on peak slope, and HPLC conditions were as described in the HPLC Fractionation section except that the H_3PO_4 buffer was omitted from the water: CH_3CN mobile phase. Peak 1 and Peak 2 fractions from 16 injections of 25 µL each were collected and combined for each peak. Triplicate samples of each peak were collected.

For the atrazine assay, Peak 1 and Peak 2 samples were evaporated to 150 µL and reacted with 30 µL of 10 mg L^{-1} atrazine in 10% CH_3OH for an initial concentration of 1.667 mg L^{-1} under conditions as previously described. Following the 16 hour reaction time, LLE was used to isolate atrazine from the sample matrix. Two milliliters of

dichloromethane were added to stop the reaction and 820 µL of water were added to facilitate separation of the two phases. The samples were then extracted an additional two times with 1 mL of dichloromethane. The organic fraction was removed, evaporated to dryness, and re-suspended in 180 µL of 2:3 CH_3CN:H_2O (v:v). Samples were analyzed with HPLC as previously described, but a 20 min isocratic method of 0.1% H_3PO_4: CH_3CN (11:9; v:v) was used. The atrazine retention time (R_t) was 9.5 min.

Results and Discussion

Degradation of Atrazine by Root Homogenates. Results from root homogenates extracted with CH_3OH or ethyl acetate confirmed the presence of atrazine-degrading phytochemicals in EG roots that are readily extracted with common solvents. The crude extracts prepared with CH_3OH degraded 41.6 ± 6.4% (standard deviation) of the added atrazine, while the ethyl acetate extracts degraded 37.3 ± 16.3% of the atrazine. Both degraded significantly more than the control. These results were very promising considering the variety of compounds that are typically present in such an unrefined root extract. The results indicated that the active atrazine degrading compound(s) were either present in high concentrations or were extremely potent in their degradation activity against atrazine.

Although mean atrazine degradation between the two solvents was not significantly different based on the results of Tukey's HSD test, the reactivity of the CH_3OH root extract was numerically greater and less variable than ethyl acetate. Additionally, in light of the study's aim to identify and characterize atrazine-degrading compounds that would be viable in soil and water, the presumably more polar compounds extracted by CH_3OH were selected for further investigation.

Degradation of Atrazine by Liquid/Liquid Extraction Fractions. Relative to the water control, aqueous fractions from the LLE degraded 23.5 ± 2.19% of the added atrazine and the organic fractions degraded 18.3 ± 2.04 % of the added atrazine. Both fractions degraded significantly more than the control, but data for the aqueous and organic fractions were not significantly different. It should be noted that the chloroform fraction was re-dissolved in water to perform the assays without solvent interference, and many or most of the compounds in this fraction likely had limited water solubility. Since the aqueous fractions exhibited greater atrazine-degrading activity, they were used for subsequent isolation and purification. Also, the most "active" compounds in the aqueous fraction were apparently polar in nature given that they were water soluble. In light of the long-term goal to develop a natural product to mitigate atrazine contamination of water resources, water insoluble phytochemicals would be of little practical significance. Therefore, only the identity of the aqueous phase compounds was pursued.

Degradation of Atrazine by SPE Fractions. Results of the SPE elution experiment showed that the 3rd and 4th SPE fractions degraded 95.8 ± 5.6% and 99.8 ± 0.32% of the atrazine, respectively (Figure 2.3). Atrazine degradation in the 5th through 8th SPE fractions ranged from 52.1to 90.6% of the added atrazine, while the 1st, 2nd, 9th, and 10th fractions degraded less than 36%. Since the 3rd through 8th fractions degraded more than 50% of the atrazine, they were selected for further fractionation and testing.

Degradation of Atrazine by HPLC fractions. Separation of the SPE fractions into 23 HPLC fractions showed that SPE fractions 3 and 4 gave rise to the two HPLC fractions with the greatest atrazine-degrading activity (Figure 2.4). Of the added atrazine, SPE fraction 3, HPLC fraction 8 degraded 68±4.2 % (range) and SPE fraction 4, HPLC

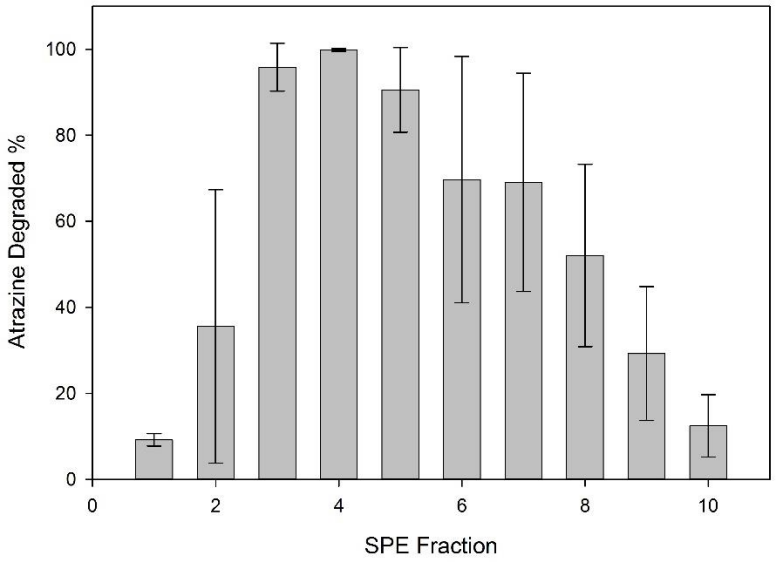

Figure 2.3 Atrazine degradation exhibited by the ten fractions collected using SPE. Error bars represent standard deviation. n= 10 for SPE fractions 3 to 8, and n=9 for SPE fractions 1, 2, 9, and 10.

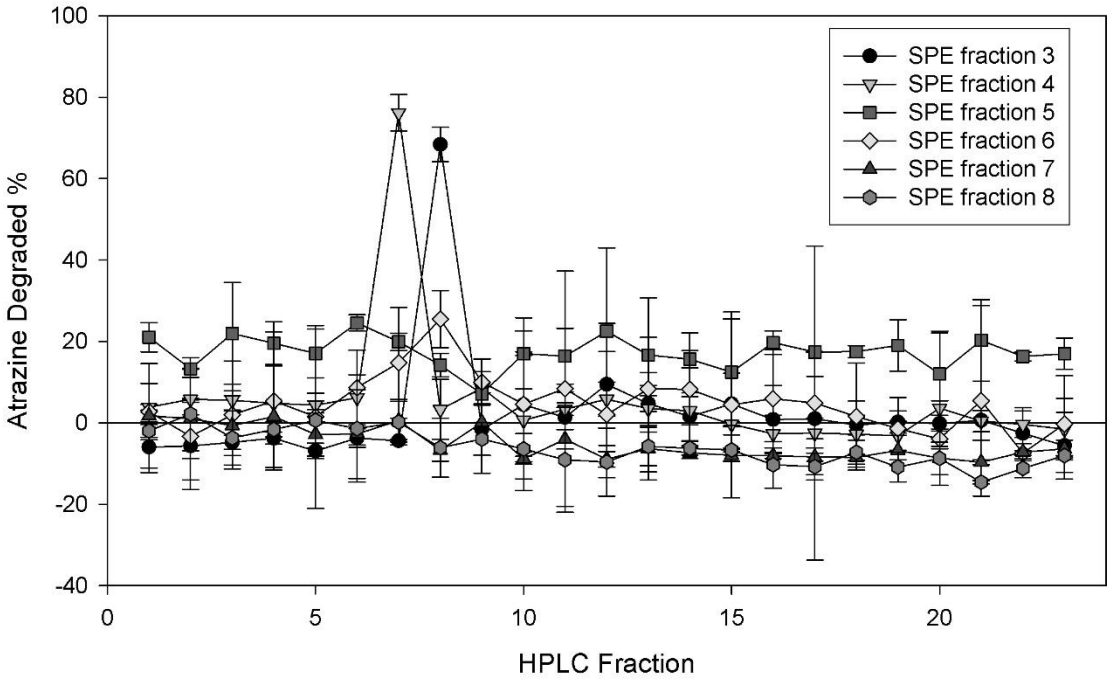

Figure 2.4 Atrazine degradation exhibited by fractions produced from HPLC separation of SPE master fractions 3 through 8. Error bars represent the range around the average. n=2

fraction 7 degraded 76±4.4 % (range) of the added atrazine. All other HPLC fractions derived from SPE fractions 3 and 4 degraded <10%, which was considered within the range of analytical error, and consequently were considered non-reactive relative to atrazine. The HPLC fractions derived from SPE fractions 5 through 8 did not exceed 33% atrazine degradation; thus, they were viewed as less promising and were not further pursued. Mass spectral data confirmed that the atrazine-degrading HPLC fractions 7 and 8 actually contained the same two compounds (Figure 2.2), and these compounds were initially designated as Peak 1 and Peak 2 prior to identification.

Mass Spectral Identification. Three separate points of identification were used to determine the chemical structures of Peak 1 and Peak 2 by comparing root extract samples to authentic Bx standards: 1) HPLC-UV R_t (Figure 2.5a and 5b); 2) LC/MS extracted ion chromatogram (XIC) R_t for the pseudomolecular ion mass (Figure 2.5c and 5d); and 3) LC/MS/MS fragment ion spectra (Figure 2.5e-5h).

The HPLC-UV chromatograms of Peak 2, with a R_t of 12.65 min, closely matched that of the main peak (R_t = 12.63 min) in the Bx hydroxamic acid standard of DIBOA-Glc (2-β-D-glucopyranosyloxy-4-hydroxy-1,4-benzoxazin-3-one) (Figures 2.5a and 5b). The XIC of Peak 2 showed a pseudomolecular ion $[M+H]^+$ of *m/z* 344, which also matched the mass of the DIBOA-Glc standard (Figures 2.5c and 5d) and that previously reported for DIBOA-Glc in the literature (Cambier et al. 1999). Product ions from the precursor ion spectra of root extract and the standard yielded *m/z* 164 and *m/z* 182 as the two most abundant ions, with relative abundances of 100% for *m/z* 164 for both extract and standard, and 81% and 69% for *m/z* 182 for the extract and standard, respectively (Figures 2.5e and 5f). Loss of the glucose moiety (180 mu) less a water

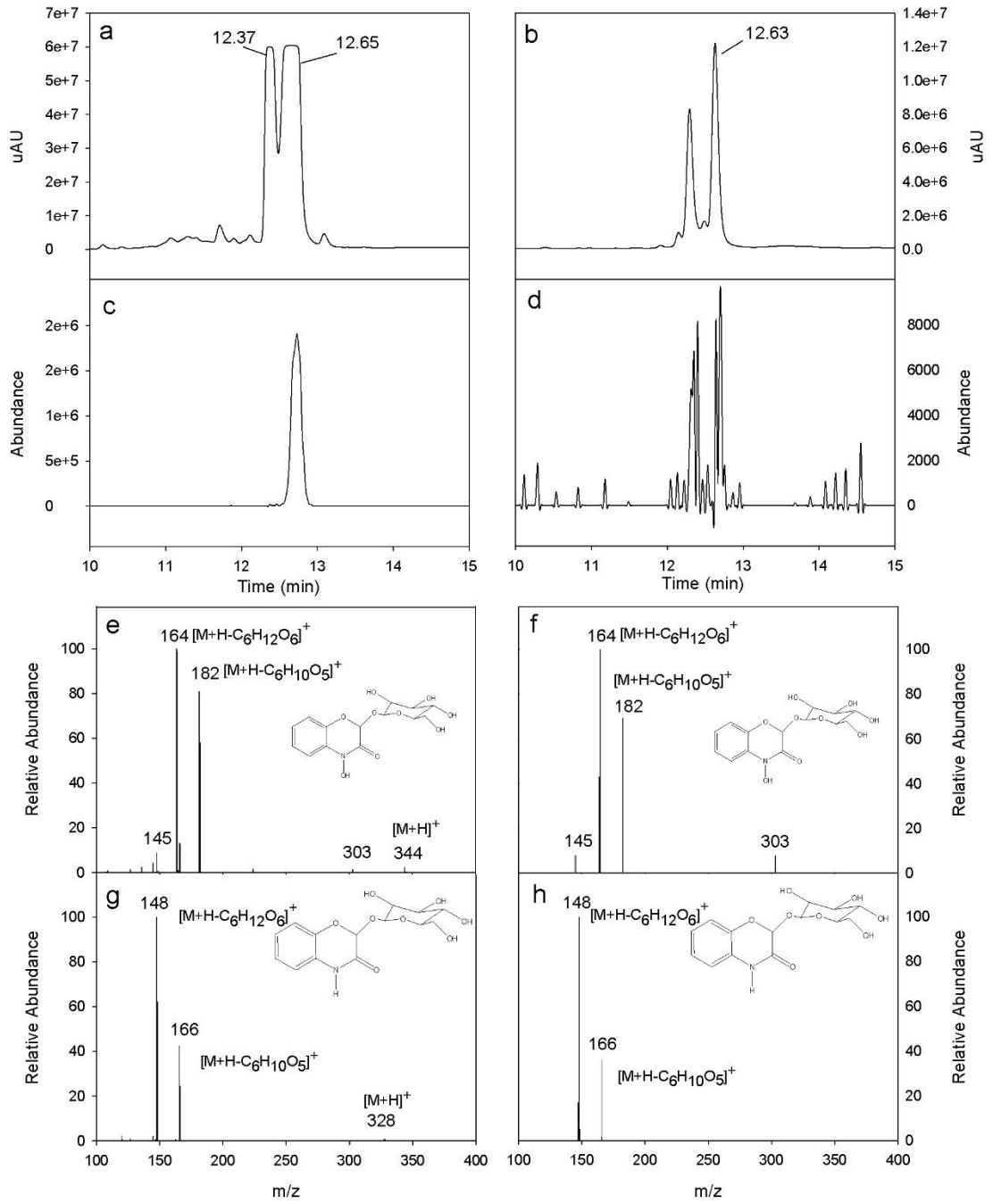

Figure 2.5 Tenative identification of Peak 1 and Peak 2 from root extract samples from a) UV chromatogram of root extracted sample (Peak 1 Rt= 12.37, Peak 2 Rt= 12.65), b) UV chromatogram from authentic DIBOA-Glc standard (Rt= 12.63), c) XIC for m/z 344 from root extracted sample, d) XIC for m/z 344 from authentic DIBOA-Glc standard, e) MS/MS spectra $[M+H]^+$ = 344 from root extracted sample, f) MS/MS spectra $[M+H]^+$ = 344 from authentic DIBOA-Glc standard, g) MS/MS spectra $[M+H]^+$ = 328 from root extract sample, h) MS/MS spectra for $[M+H]^+$= 328 from HBOA-Glc authentic standard.

molecule from the *m/z* 344 precursor ion resulted in the *m/z* 182 ion and was tentatively identified as $[M+H-C_6H_{10}O_5]^+$. The *m/z* 164 ion represented the loss of the glucose moiety from the precursor ion and was tentatively identified as $[M+H-C_6H_{12}O_6]^+$. The precursor ion spectra of the Peak 2 root extracts closely matched that of the DIBOA-Glc standard in terms of the ions present in the spectra (*m/z* 145, 164, 182, and 303) and similar relative ion abundances. The relative ion abundances for the root extracts and DIBOA-Glc standard were 4.3% and 7.9% for *m/z* 145, and 1.4% and 7.8% for *m/z* for 303, respectively. Based on the data in Figures 2.5a-5f, the compound in Peak 2 was identified as DIBOA-Glc (2-β-d-glucopyranosyloxy-4-hydroxy-1,4-benzoxazin-3-one).

In the root extracts and the authentic material, the HPLC-UV chromatogram of Peak 1 showed a R_t of 12.37 minutes (Figure 2.5a), and the LC/MS XIC for Peak 1 (not shown) had a pseudomolecular ion, $[M+H]^+$, of *m/z* 328 at the same R_t. The pseudomolecular ion mass also corresponded to data in the literature for the benzoxazinoid lactam HBOA-Glc (Cambier et al. 1999). The fragment ion spectrum for Peak 1 was very similar to the spectra of DIBOA-Glc (Figures 2.5e and 5g), which is the corresponding hydroxamic acid to HBOA-Glc and differs by only a hydroxyl group. Moreover, the fragment ion spectra of root extracts and the HBOA-Glc standard showed that ions *m/z* 166 and 148 were the dominant ions resulting from fragmentation of the *m/z* 328 precursor ion, with relative abundances of 42% and 36% for *m/z* 166, and 100% for *m/z* 148, for the extract and authentic material, respectively (Figures 2.5g and 5h). These fragments were analogous to the pattern observed in the DIBOA-Glc spectra, with *m/z* 166 representing the loss of glucose less a water molecule $[M+H-C_6H_{10}O_5]^+$ and *m/z* 148 representing loss of the glucose moiety $[M+H-C_6H_{12}O_6]^+$. On the basis of these three

points of identification (HPLC-UV R_t, LC/MS XIC, and fragment ion spectra) Peak 1 was identified as HBOA-Glc (2-β-d-glucopyranosyloxy-1,4-benzoxazin-3-one).

Concentrations in EG Root Tissue. The estimated fresh weight concentration of DIBOA-Glc was 210±28 mg kg^{-1} and the HBOA-Glc concentration was 71 ±19 mg kg^{-1} (dry wt. basis 710±96 mg kg^{-1} and 240±74 mg kg^{-1}, respectively) (note: UV data in Figure 2.5a represents 1/5th of this concentration as the sample was diluted 1/5 prior to injection). The estimated concentrations showed that DIBOA-Glc in EG root tissue was approximately three times more concentrated than HBOA-Glc and both compounds were present in mM concentrations (DIBOA-Glc 3.46 ±0.02 mM and HBOA-Glc 1.25 ± 0.42 mM).

In the peer-reviewed literature, reports of Bx compounds in EG tissues are limited to Bx occurrence and do not include concentration estimates. Bx compound concentration data for other species compared to those reported here showed that EG root tissue has orders of magnitude lower Bx concentrations (Hofman and Hofmanova 1969, Lyons et al. 1988, Gianoli and Niemeyer 1998, Baumeler et al. 2000, Larsen and Christensen 2000). However, in some cases the range of reported concentrations for Bx compounds spans four orders of magnitude even from leaves of the same plant (Lyons et al. 1988). Additionally, the methods employed for extraction in the current study were not optimized for Bx recovery, but were intentionally generic to facilitate a broad search for novel atrazine-degrading phytochemicals in EG. The broad range of extraction methods, plant species, plant growth stages, plant tissues, and wet weight versus dry weight basis used for reporting Bx concentrations also impedes direct and meaningful comparisons of our results to those reported in the literature. Nevertheless, the estimated concentrations reported here provide the first estimates of specific Bx compounds in EG root tissue.

Since the late 1980's, several studies in the literature have reported the occurrence of Bx compounds in *Tripsacum* (Niemeyer 1988, Marcacci et al. 2006, Frey et al. 2009). These references claim Bx occurrence in *Tripsacum* in the context of demonstrating widespread occurrence of the compounds across species, and not in the context of research objectives to establish the information as authentic. However, further investigation of the references cited in these papers revealed that these claims were not supported by rigorous, peer-reviewed study. A reference supplied in the Niemeyer (1988) review cited a 1976 newsletter reporting identification of three Bx compounds in EG shoots. While the newsletter report may have been trustworthy, the data was never confirmed or reported in a peer-reviewed article. Later articles citing the Niemeyer (1988) review as the source for claiming Bx occurrence in *Tripsacum* likely took for granted the original source of the data. The source of information from which Frey et al. (2009) claims DIBOA/DIMBOA as the dominant Bx compounds in *Tripsacum* was also unclear. These examples lead to the conclusion that the occurrence of Bx compounds in EG had not been rigorously substantiated in the peer-reviewed literature. As such, this study represents the first definitive identification of Bx compounds in EG roots and the first estimated concentration of Bx compounds in the *Tripsacum* genus.

Atrazine-Degrading Activity of HBOA-Glc and DIBOA-Glc. The HBOA-Glc (Peak 1) samples showed negligible degradation with 1.7 ± 3.2% of the added atrazine degraded. DIBOA-Glc (Peak 2) samples degraded an average of 23.7±11.8% of the added atrazine. The results clearly establish DIBOA-Glc as the atrazine-degrading compound as the activity of HBOA-Glc was shown to be negligible. HBOA-Glc and DIBOA-Glc are structurally related Bx compounds, but their reactivity towards atrazine was markedly

different. The presence of the nucleophilic hydroxamic acid moiety in DIBOA-Glc was apparently critical to its reactivity towards atrazine while the lactam moiety was shown to be unreactive. These results suggest that DIBOA-Glc was at least partially responsible for the enhanced rhizosphere degradation of atrazine in EG observed by Lin et al. (2011). While the aim of the study was not to perform a comprehensive survey of all Bx compounds present in *Tripsacum*, it demonstrated that EG is a natural source of an atrazine-degrading phytochemical that could lead to new strategies for reducing and remediating atrazine contamination of water resources.

Willett, C. D., R. N. Lerch, K. W. Goyne, N. D. Leigh, C. H. Lin and C. A. Roberts (2014). A simple method for isolation and purification of DIBOA-Glc from *Tripsacum dactyloides*. *Natural Product Communications* **9**(9): 1283-1286. Reproduced by permission of Natural Product Communications.

CHAPTER 3: A SIMPLE METHOD FOR ISOLATION AND PURIFICATION OF DIBOA-GLC FROM *TRIPSACUM DACTYLOIDES* (L.)

Abstract

Naturally occurring benzoxazinones (Bx) are a highly reactive class of compounds that have received particular attention in the past several decades. Recently, we identified 2-β-D-glucopyranosyloxy-4-hydroxy-1,4-benzoxazin-3-one (DIBOA-Glc) as the compound present in the roots of Eastern gamagrass (*Tripsacum dactyloides* (L.)) responsible for atrazine degradation. However, characterization of the DIBOA-Glc/atrazine degradation reaction has been limited due to difficulties in attaining sufficient quantities of purified DIBOA-Glc. The objective of the study was to develop a simple purification and isolation method for obtaining bulk quantities of highly purified DIBOA-Glc. *T. dactyloides* roots were extracted with 90% aqueous methanol, and the crude extract was fractionated using an HPLC equipped with a C_8 semi-prep column and fraction collector. UHPLC-DAD-MS/MS was used to confirm the identity of DIBOA-Glc in the fractions collected. Analysis by ^{13}C and ^{1}H NMR and DAD indicated that 542 mg of DIBOA-Glc with a purity of >99% was obtained. The reactivity of the DIBOA-Glc was confirmed in a 16 hour assay with atrazine, which resulted in 48.5% ± 1.2% (SD) atrazine degradation. The method described here offers several advantages over existing extraction and

synthesis methods, which are more cumbersome, use hazardous chemicals, and yield only small quantities of purified compound. The newly developed method will facilitate future research characterizing the chemical behavior of DIBOA-Glc and determine its potential as an atrazine mitigation and remediation tool.

Introduction

Naturally occurring benzoxazinones (Bx) have been studied extensively over the last several decades and are highly reactive natural compounds (Sicker and Schulz 2002). While early work focused on their ability to impart triazine-resistance in a number of agronomically important crops (Castelfranco et al. 1961, Castelfranco and Brown 1962, Hofman and Hofmanova 1969), recent work has focused on their use as naturally occurring herbicides in rye (*Secale cereale*) cover crops (Rice et al. 2012). Research has shown that Bx compounds possess a wide range of other beneficial properties including insecticidal, anti-tumor, anti-fungal, and anti-microbial properties (Zhang et al. 1995, Sicker and Schulz 2002, Sánchez-Moreiras et al. 2003, Niemeyer 2009). Recently, we identified 2-β-D-glucopyranosyloxy-4-hydroxy-1,4-benzoxazin-3-one (DIBOA-Glc) (Figure 3.1) as the atrazine-degrading compound present in the roots of Eastern gamagrass (*Tripsacum dactyloides* (L.)) (Willett et al. 2013). *T. dactyloides* is a native grass species used in vegetative filter strips as a conservation measure to mitigate surface water contamination in agroecosystems. However, characterization of the DIBOA-Glc /atrazine degradation reaction has been limited due to difficulties in attaining sufficient quantities of purified DIBOA-Glc.

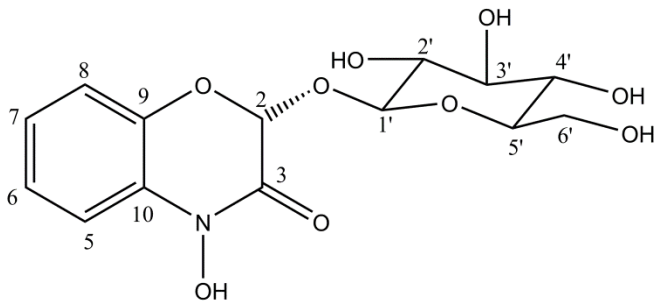

Figure 3.1. 2-β-D-glucopyranosyloxy-4-hydroxy-1,4-benzoxazin-3-one (DIBOA-Glc).

Currently, DIBOA-Glc cannot be readily purchased from commercial sources, and therefore must be extracted or synthesized. Of the numerous procedures available for obtaining the compound, the need for a simplified purification and isolation method for obtaining bulk quantities of highly purified DIBOA-Glc remains. Such a method would use readily available solvents of low toxicity, require little sample preparation, and would use common chromatographic instruments to partially automate the separation process. Many of the available methods are sufficient for producing highly purified material suitable for preparing quantitative standards (Bailey and Larson 1989, Cambier et al. 1999, Sue et al. 2000), but these methods do not produce sufficient DIBOA-Glc mass required of larger scale studies. Furthermore, these published methods often lack information regarding purity, yield, identification confirmation, and employ complicated extraction or synthesis schemes. One group has developed a method for obtaining highly purified material from plant extracts (Yin et al. 2008), but the use of an uncommon high-speed counter-current chromatography (HSCCC) system limits it widespread applicability. Hartenstein and Sicker (1994) reported the isolation of 350 mg of DIBOA-Glc from rye shoots by successive solvent extraction followed by TLC separation. However, the method is labor intensive and not conducive to automation. Another approach for obtaining pure Bx compounds involves the use of stereoselective synthesis

(Kluge and Sicker 1996). While this method can synthesize highly pure DIBOA-Glc, the procedure yields <100 mg of purified material and requires the use of hazardous components. The current research demonstrates a purification method using HPLC with direct injection of crude aqueous methanol root extracts from *T. dactyloides* to yield substantial mass of purified DIBOA-Glc.

Results and Discussion

Aqueous methanol extracts of *T. dactyloides* roots were fractionated using HPLC with a semi-prep column and fraction collector. A sample chromatogram is shown in Figure 3.2a. A large injection volume (500 μL) created an overload of the column, resulting in poor peak shape. However, the peak of interest exhibited a consistent retention time (15.7 min). Figure 3.2b shows a sample chromatogram of the purified material diluted to 20 μM as analyzed using HPLC with an analytical column. The purified sample contained a single peak at 16.8 min, qualitatively demonstrating the robust utility of the semi-prep column purification procedure.

To confirm the identity and purity of the collected factions, several spectrometric techniques were employed including ^{1}H and ^{13}C NMR, and UHPLC with DAD, and MS/MS. The 2-D NMR spectra confirmed the identity of DIBOA-Glc in the sample containing the collected fraction. The ^{1}H and ^{13}C NMR spectra confirmed the number of H and C atoms and bonds in the molecule. The arrangement of H and C atoms and bonds in the molecule as determined by ^{1}H-correlation spectroscopy (^{1}H –COSY) and heteronuclear multiple-quantum correlation (HMQC) analyses (data not shown) were consistent with the expected results for the DIBOA-Glc molecule.

The UV spectra obtained by UHPLC-DAD for t_R= 2.2 min corresponded with previously reported UV (methanol) λ_{max} 202, 259, and 287 for DIBOA-Glc (Baumeler et al. 2000). The chromatographic peak with t_R= 2.2 min showed a pseudomolecular ion $[M+H]^+$ of m/z 344, which fragmented to yield product ions at m/z 164 and 182, with relative abundances of 100% and 19%, respectively; this was in agreement with previously reported spectra for DIBOA-Glc (Cambier et al. 1999, Willett et al. 2013). The 2-D NMR, UV-Vis spectra, and MS/MS data provided five points of identification.

The DIBOA-Glc peak dominated the UV-Vis spectrum. A small peak with t_R =0.9 min was tentatively identified as acetone based on the UV spectrum (λ_{max} = 264 nm). The trace amount of acetone was presumed to be from glassware solvent rinsing and was considered a sample matrix component and not an impurity. Thus, the dominant peak at t_R = 2.2 min in the UHPLC-DAD chromatogram was the only peak present, resulting in a purity estimate of >99%.

All peaks present in the ^{1}H and ^{13}C spectra were identified as sample matrix components or the target compound, thus yielding a purity of >99% for the isolated DIBOA-Glc. The process yielded 0.64 mg of DIBOA-Glc per 500 µL injection. In total, 542 mg of highly pure (>99%) material was collected from approximately 4 kg of roots (wet weight). In comparison, the HSCCC method used by Yin et al. (2008) resulted in 4.8 mg of DIBOA-Glc (94.8% purity) from 1 kg of dry aerial plant parts. A synthesis method developed by Kluge and Sicker (1996) yielded 27 mg of DIBOA-Glc with a quantitative measure of purity not reported. Hartenstein and Sicker (1994) isolated 350 mg from 1 kg of rye shoots, but a quantitative measure of purity was not reported. These synthesis and extraction methods yielded only a fraction of what was obtained with the current method.

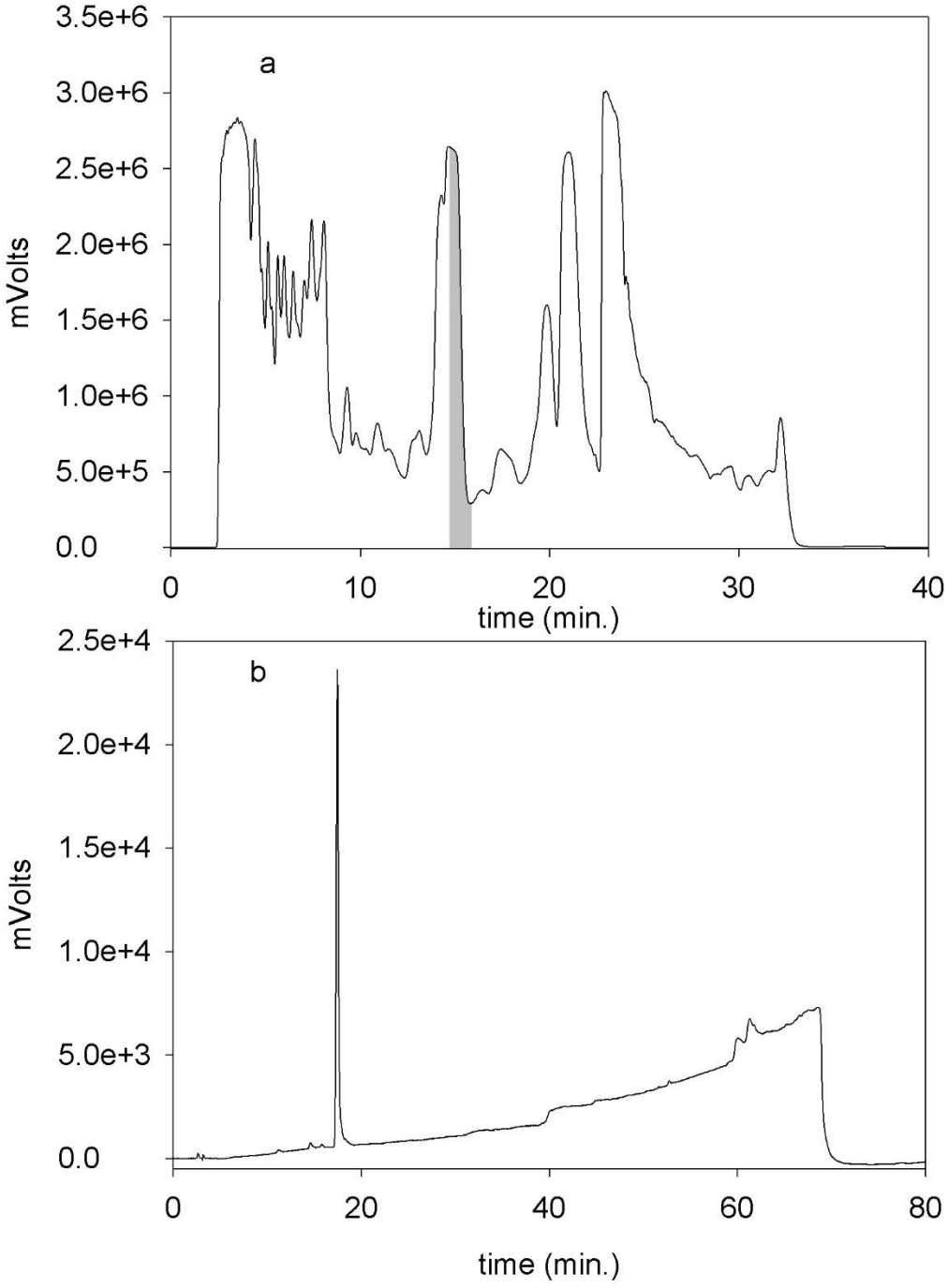

Figure 3.2. Chromatographs of a) crude methanol root extracts with the semi-prep column method, shaded area is the fraction collected and b) purified DIBOA-Glc material diluted to 20 µM and analyzed with a Columbus 250x4.6mm, 5µm, C_8, 110Å analytical column and 25 µL injection volume. Mobile phase was 1 mL/min methanol/water with a gradient from 10-75% methanol over 65 min, held at 10% methanol from 65-80 min.

To demonstrate the reactivity of the extracted and isolated DIBOA-Glc, a simple assay was performed with atrazine for 16 hours. The result was 48.5 ± 1.2% (SD) degradation of the atrazine. The degradation of atrazine in the presence of the isolated DIBOA-Glc was consistent with previous findings (Willett et al. 2013).

The results of the identification confirmation and purity determination demonstrate that a simple methanol extraction procedure followed by HPLC semi-prep separation produced highly purified material sufficient to use as an authentic standard material for quantitative research. The goal of the current study was to obtain highly purified material in the most facile way, and Figure 3.2 demonstrates the efficacy of the method and its ability to eliminate numerous compounds within the crude root extract to that of a single highly purified compound. The method offers advantages of simplicity, use of standard HPLC equipment, and compound selectivity. With minor changes based on compound retention time, this utilitarian method could be adjusted to collect any compound of interest that is compatible with the C8 column and mobile phase system. Possible applications include isolation of other Bx compounds or phytochemicals from a variety of plant species or tissues. However, the method is not without its limitations. Because crude methanol root extracts were injected directly on to the column, a lengthy HPLC method was required to sufficiently separate compounds and maintain the integrity of the column. As such, the current method was time consuming. A total of 24 days of instrument time were required to collect 542 mg of DIBOA-Glc. Accounting for the additional time required for concentrating the HPLC fractions, this is comparable to the 8 hours needed by Yin et al. (Yin et al. 2008) to collect just 4.6 mg of DIBOA-Glc. In addition, large volumes of solvent were required to operate the pumps at the 4 mL min^{-1}

flow rate; approximately 27 L of methanol were used for the HPLC separations. But methanol can be obtained in high purity for relatively low cost, and is considered a better alternative to other more expensive and environmentally hazardous solvents commonly used in HPLC. While standard HPLC equipment was used for the separation, the initial identification and purity determinations utilized MS and NMR analyses, which are less widely available than standard HPLC instrumentation. However, these analyses are only needed initially to establish compound purity and commercial or research laboratories can provide these analyses on a contract basis. The newly developed isolation and purification method, which requires minimal sample preparation, employs non-chlorinated solvents, and is conducive to automation with standard HPLC instrumentation, will facilitate future research to characterize the reaction mechanism of the DIBOA-Glc/atrazine reaction and help to determine DIBOA-Glc's potential as a mitigation and remediation tool.

Experimental

Chemicals. Chromatography grade solvents and reagents including acetonitrile, ammonium formate, dichloromethane, methanol, and concentrated phosphoric acid were purchased from Fisher Scientific (Pittsburg, PA, USA). Atrazine (98.9%) was purchased from ChemService (West Chester, PA, USA). Dimethyl sulfoxide-d6 (DMSO-d6, 99.96% atom D) was purchased from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA, USA). Pyridine (99.5%) was purchased from Alpha Aesar (Ward Hill, MA, USA). Ultrapure chromatography grade water was used for sample preparation and HPLC mobile phases.

Extraction and Fractionation. *T. dactyloides* root material was collected and methanol root extracts were prepared as described previously in Willett et al. (2013). In brief, *T. dactyloides* roots were homogenized in 90% methanol/water using a blender and then extracted on an end-to-end shaker for 12-16 hours. Root extracts were filtered to remove solid material and extracted with an additional 100 mL methanol. The filtrate was evaporated to achieve a 10% methanol/water solution (for antimicrobial purposes). Samples were then centrifuged for 20 min at 5000 rpm and 10 °C to further remove colloids and other solids from the samples. This supernatant was then directly injected into an HPLC system for purification.

A Shimadzu LC-10AT VP (Columbia, MD, USA) HPLC system equipped with an auto injector, fraction collector, UV detector, and a Phenomenex Luna C8 (2) (250 x 50 mm) semi-prep column (Torrance, CA, USA) were used to purify DIBOA-Glc. An isocratic method using a 20% aqueous methanol (1:4 CH_3OH:H_2O) mobile phase at a flow rate of 4 mL min^{-1} for 40 min with UV detection at 220 nm and a sample injection volume of 500 µL was employed. The retention time of DIBOA-Glc was 15.7 min. Fraction collection was based on slope/threshold parameters indicated for a 2 min time window between 15-17 min, and parameters were set to reduce contamination from nearby peaks. Eight-hundred forty-eight fractions were collected. The fractions were then combined, evaporated, and solvent exchanged to produce a concentrated stock solution of 192 mL in 80% methanol.

Identification Confirmation. An 8 mL aliquot of the 80% methanol stock solution was evaporated to dryness under a stream of N_2 gas and re-suspended in 650 µL of DMSO-d_6. The sample was centrifuged and transferred into a 500 MHz NMR sample tube. A Bruker

DRX 500 MHz spectrometer with a cryo-chilled TCI probe was used to acquire ^{1}H and ^{13}C spectra (Billicera, MA, USA). Compound structure was further confirmed using 2-D NMR ^{1}H- correlation spectroscopy (COSY) and heteronuclear multiple-quantum correlation (HMQC) data analyses.

A 20 μM aqueous solution of DIBOA-Glc was analyzed for identity with a Waters (Milford, MA, USA) UHPLC-DAD-MS/MS. An ammonia formate/methanol gradient mobile phase was used to analyze 10 μL injection volumes. The mobile phase was 2% methanol from 0-4 min, 100% methanol from 4-4.3 min, and 2% methanol from 4.4-5 min. The flow rate was 0.4 mL min^{-1}. A Waters C_{18} (1.7 μm, 2.1 x 50 mm) column was used at 40 °C. These conditions resulted in a retention time for DIBOA-Glc of 2.2 min. Photodiode array spectra were collected from 190-450 nm, and the UV-VIS spectrum was used as an additional point of identification. For the mass spectroscopy, ESI (+) was the ionization method. Chromatograms and spectra were compared against a previously obtained DIBOA-Glc authentic standard (Willett et al. 2013) to confirm the identity of DIBOA-Glc in the sample.

Purity Determination. Two approaches were used for purity determination. One involved use of the UHPLC-DAD, in which purity was determined as area of the DIBOA-Glc peak divided by the total area of all peaks in the chromatogram scan from 190-450 nm. The other method was NMR in which the peak integrations of unidentified peaks in the ^{1}H and ^{13}C spectra were used in a similar manner to calculate the purity of the NMR sample material (Bharti and Roy 2012).

Concentration Determination. To determine DIBOA-Glc concentration in solution, 5 μL (0.00491 g) of pyridine (99.5% purity) was added to 550 μL (0.65416 g) of the sample

used for NMR analysis (DIBOA-Glc dissolved in DMSO-d6). The 2-D NMR analyses were repeated as described above. Peak intensities of pyridine and the sample were compared to determine concentration. Four combinations of peak intensities were assessed and the reported concentration represents the mean value. An assumed sample purity of 100% was used during the calculations of concentration as no unidentified peaks were present in the spectra.

Atrazine Degradation Confirmation. A simple assay was carried out to confirm the atrazine-degrading ability of the isolated extract. Reaction vessels containing 500 µL of 2000 µM DIBOA-Glc and 500 µL of 20 µM atrazine were allowed to react at ambient temperature for 16 hours. Control samples containing 500 µL DI water and 500 µL of 20 µM atrazine were also tested. Reaction samples and controls were prepared in triplicate. Liquid/liquid extraction with 1 mL dichloromethane x 3 was performed to stop the reaction. The organic fraction was collected, evaporated to dryness under a stream of N_2 gas at 40 °C, and resuspended in 1 mL of 40% acetonitrile/water. Atrazine concentration was analyzed using HPLC as described in Willett et al. (2013).

Spectra for 2-β-D-glucopyranosyloxy-4-hydroxy-1,4-benzoxazin-3-one (DIBOA-Glc).

UV/Vis λ_{max} (water) nm: 212, 255, and 280

^{1}H NMR (500 MHz, DMSO-d_6) δ: 7.26 (2H, dd, *J*= 7.9, 0.9 Hz, H-5), 7.12 (2H, m, H-6), 7.09 (2H, m, H-8), 7.02 (2H, td, *J*= 7.6, 1.2 Hz, H-7), 5.90 (1H, s, H-2), 4.58 (1H, dd, *J*= 7.9 Hz, H-1'), 3.68 (2H, dd, *J*= 11.6, 1.4 Hz, H-6'), 4.10 (1H, br s, N-OH), 3.03 (1H, t, *J*= 9.2 Hz, H-4'), 2.91 (1H, t, *J*= 8.4 Hz, H-2').

^{13}C NMR (500 MHz, DMSO-d_6) δ: 155.5 (C, C-3), 140.5 (C, C-9), 128.5 (C, C-10), 124.0 (C, C-7), 122.9 (C, C-6), 117.6 (C, C-8), 112.9 (C, C-5), 102.7 (CH, C-1'), 96.3 (CH, C-2), 77.5 (CH, C-5'), 76.8 (CH, C-3'), 73.3 (CH, C-2'), 69.7 (CH, C-4'), 61.0 (CH_2, C-6').

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CHAPTER 4- CHARACTERIZATION OF THE BENZOXAZINONE-TRIAZINE DEGRADATION REACTION USING DIBOA-GLC AND ATRAZINE

Abstract

The role of benzoxazinones (Bx, 2-hydroxy-2*H*-1,4-benzoxazin-3(4H)-one)) in triazine degradation and resistance has been studied for over half a century. In this research, the fundamental parameters of the reaction between DIBOA-Glc (2-β-D-glucopyranosyloxy-4-hydroxy-1,4-benzoxazin-3-one) and atrazine (ATR, 6-chloro-*N*-ethyl-*N'*-(1-methylethyl)-1,3,5-triazine-2,4-diamine) were examined. Through a series of experiments employing a variety of chromatographic and spectroscopic techniques, the DIBOA-Glc-ATR reaction was characterized in terms of reactant and product kinetics, stoichiometry, identification of a reaction intermediate, reaction products formed, and pH effects. Results of these experiments demonstrated that the reaction mechanism proceeds via nucleophilic attack of the hydroxamic acid moiety of DIBOA-Glc at the C-2 position of the triazine ring, forming hydroxyatrazine (HA, 2-hydroxy-4-ethylamino-6-isopropylamino-*s*-triazine), with associated degradation of DIBOA-Glc. The degradation of reactants followed 1st-order kinetics with a non-catalytic role of DIBOA-Glc, and the reaction rate increased with decreasing solution pH. A reaction intermediate was identified as a DIBOA-Glc-HA conjugate, indicating a 1:1 DIBOA-Glc:ATR stoichiometry. Reaction products included HA and Cl^-, but definitive identification of DIBOA-Glc reaction product(s) was not attained. With these reaction parameters

elucidated, DIBOA-Glc can be evaluated in terms of its potential for a myriad of applications, including its use to address the problem of widespread ATR contamination of water resources.

Introduction

The reaction of benzoxazinoid (Bx, 2-hydroxy-2*H*-1,4-benzoxazin-3(4H)-one) compounds with chloro-triazine herbicides has been studied for over half a century (Castelfranco et al. 1961, Niemeyer 1988, Raveton et al. 1997, Willett et al. 2013). The early researchers studying the fundamental aspects of the chemical reaction were able to make remarkable advances in knowledge with limited technology and lack of pure Bx standards. Much of the primary work focused on identifying the causative agent of triazine resistance in various plants and determining the fate and weed control efficacy of triazine herbicides (Castelfranco et al. 1961, Hamilton and Moreland 1962, Hamilton 1964). As research continued, applied science studies became the focus of research, and detailed characterization of the Bx-triazine reaction was not revisited.

This particular set of investigations will focus on the reaction occurring between atrazine and DIBOA-Glc (2-β-D-glucopyranosyloxy-4-hydroxy-1,4-benzoxazin-3-one). Atrazine is used extensively across the Midwest for broadleaf control in corn (Blanchard and Donald 1997, Battaglin et al. 2003, Lerch and Blanchard 2003, Lerch et al. 2008). DIBOA-Glc was recently identified as the atrazine degrading component in eastern gamagrass (*Tripsacum dactyloides* (L.) L., EG) (Willett et al. 2013), which is an important forage grass that has potential for use as a phytoremediation species (Lin et al. 2004, 2008, 2011).

Early studies showed that Bx compounds possessing hydroxamic acid moieties convert triazine herbicides to their hydroxylated metabolites (Castelfranco et al. 1961, Castelfranco and Brown 1962, Hamilton and Moreland 1962, Hamilton 1964, Tipton et al. 1971, Nakano et al. 1973, Raveton et al. 1997, Wenger et al. 2005). While the formation of hydroxylated triazines as a reaction product remains undisputed, other aspects of the reaction including the formation of a reaction intermediate, fate of Cl^-, the role and fate of the Bx reactant, and to some extent the influence of pH on the reaction rate remainare unresolved.

In a seminal paper by Castelfranco et al. (1961), the reaction between simazine (2-chloro-4,6-ethylamine-*s*-triazine) and corn (*Zea mays* L.) leaf extracts was demonstrated to be a non-enzymatic detoxification route for triazines in plants. Even before the active compound responsible for the degradation was elucidated, Castelfranco et al. (1961) speculated the reaction mechanism resulting in formation of hydroxysimazine (2-hydroxy-4,6-ethylamine-*s*-triazine) involved a nucleophilic attack at the C-2 position of the triazine, leading to the formation of a triazine conjugate and release of Cl^- (Figure 4.1). Although no data on the phytochemical degradation was collected at the time, it was speculated that the Bx compound was not consumed in the reaction and thereby implied a catalytic role for the phytochemical. In a follow-up study (Castelfranco and Brown 1962), an attempt was made to characterize the phytochemical with an extensive investigation using model compounds to approximate the nature of the phytochemical in the reaction with simazine. Even without specific knowledge concerning the phytochemical structure, the hypothesized nucleophilic mechanism was upheld due to the similarities of the reaction with other nucleophiles such as pyridine and

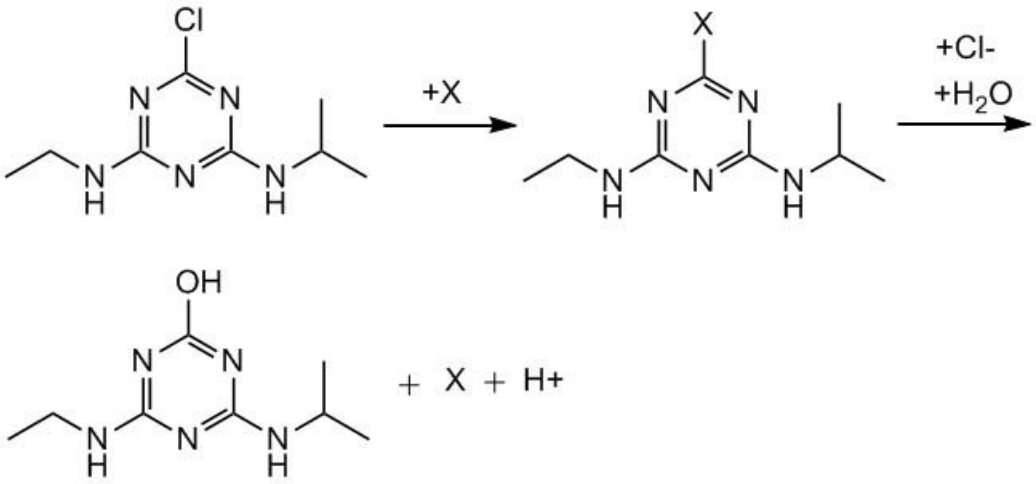

Figure 4.1 Hypothetical mechanism for Bx-catalyzed atrazine hydroxylation by corn root extracts (adapted from Castelfranco et al. 1961).

hydroxylamine. During the preparation of their manuscript, another group of researchers identified the active component of corn extracts to be the hydroxamic acid Bx compound DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one) (Castelfranco and Brown 1962).

The reaction intermediate hypothesized by Castelfranco et al. (1961) was later revisited by Nakano et al. (1973) using N-hydroxysuccinimde as a model Bx compound reacted with cyprazine (2-chloro-4-cyclopropylamino-6-isopropylamino-*s*-triazine). The proposed structure of the intermediate entailed displacement of Cl^- and joining of the two reactants by formation of a R′-**N-O-C**-R bond at C-2 of the triazine ring, where R′ = succinimide ring and R = triazine ring. However, the fate of Cl^- and the products formed from the model compounds were not characterized in the studies by Nakano et al. (1973) or Castelfranco et al. (1962). Evidence of the conjugate structure was given by NMR collected for a salt extracted and precipitated from the reaction mixture that hydrolyzed to hydroxy-cyprazine and hydroxysuccinimide in water as indicated by thin-layer

chromatography (TLC). While this provides initial evidence of formation of a reaction intermediate in the reaction between triazines and hydroxamic Bx compounds, this result has not been reproduced using naturally occurring Bx compounds.

The formation of Cl^- as indicated in Figure 4.1 received little attention after Castelfranco's initial suggestion. Raveton et al. (1997) isolated DIMBOA from corn seedlings and demonstrated that it converted atrazine to hydroxyatrazine (HA, 1-hydroxy-3-ethylamino-5-isopropylamino-2,4,6-triazine). An alternate reaction mechanism was proposed in which a chlorinated Bx product was formed. An adapted illustration of Raveton et al.'s proposed mechanism is in Figure 4.2, where the N in the

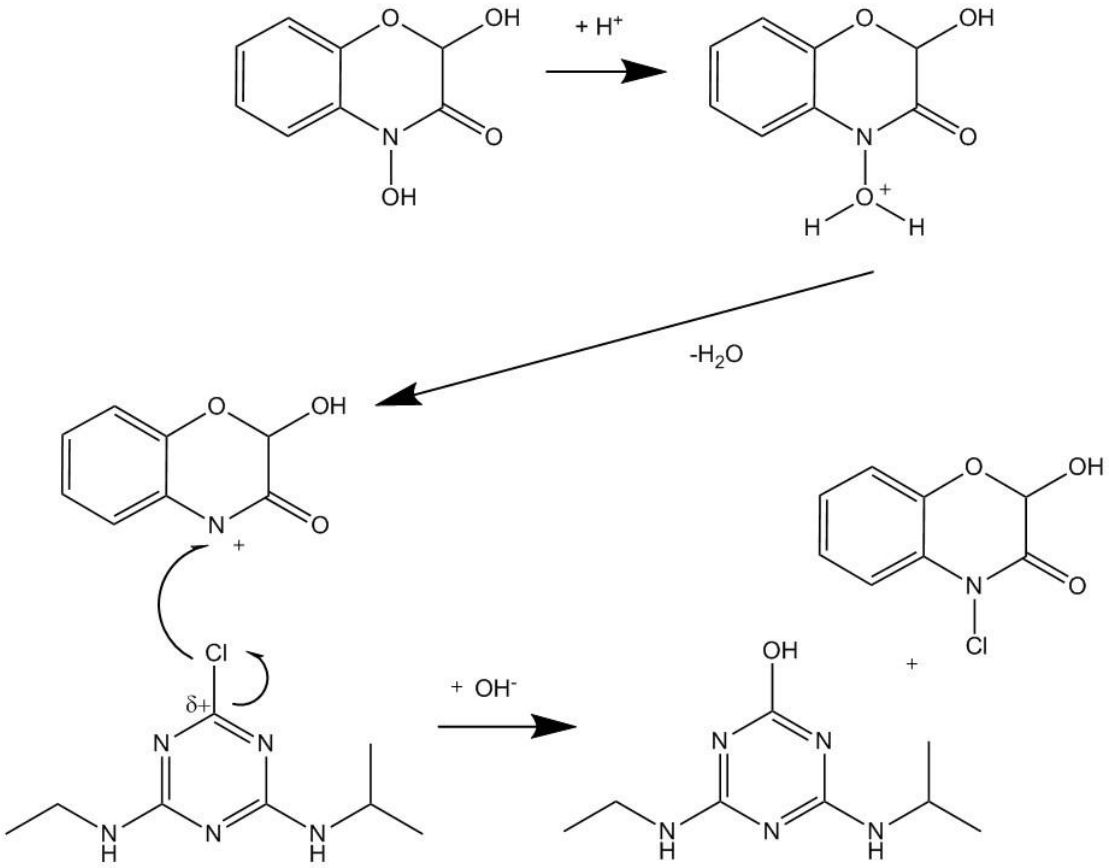

Figure 4.2. Hypothetical mechanism for atrazine hydroxylation by benzoxazinones where the Bx compound is protonated previous to reacting with atrazine and forms a chloro-benzoxazinoid product (adapted from Raveton et al. 1997).

hydroxamic acid moiety of DIMBOA is initially protonated, leading to the loss of H_2O. The resulting positively charged N^+ then becomes the electrophile reacting with the electronegative Cl^- of atrazine, forming HA and a chlorinated Bx compound. Not only does this offer a contrasting view to Castelfranco's suggested fate of Cl^- (Figure 4.1), the mechanism implies a different fate for the Bx reactant than had been hypothesized previously. The reaction was not monitored for formation of chloride, and the formation of a chlorinated Bx compound has never been detected (Raveton et al. 1997, Wenger et al. 2005).

Hamilton and Moreland (1962) presented evidence suggesting that Bx compounds behaved as catalysts in the hydroxylation of triazines. Ultra-violet (UV) absorbance was used to monitor the reaction between DIMBOA and simazine, and the results indicated no loss of DIMBOA during the reaction. Using thin-layer chromatography (TLC) to analyze the progress of the DIMBOA-simazine reaction, Tipton et al. (1971) likewise concluded that DIMBOA had not degraded during the reaction. Moreover, Nakano et al. (1973) interpreted their findings of an overall 2^{nd} order reaction rate that was independent of initial concentration of the reactants, cyprazine and N-hydroxysuccinimide, as an indication of catalytic action. The reaction of Bx compounds with diazinon, an organophosphate insecticide, is also thought to be catalytic (Ioannou et al. 1980).

In contrast, Raveton et al. (1997) investigated atrazine in reaction with various Bx compounds including DIMBOA, DIBOA (2,4-dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one), and their glucosides. Based on data indicating 1^{st} order degradation of atrazine, Raveton et al. (1997) concluded that the reaction was not catalytic and discounted Hamilton and Moreland's hypothesis. However, as pointed out in the 2009 review by Niemeyer, this

has not been considered definitive evidence, and the role of Bx compounds as catalysts for the hydroxylation of triazines remains unclear (Niemeyer 2009).

Given that a catalytic role of the Bx reactant has been the prevailing theory for several decades, little work has been done to elucidate the fate of Bx in the reaction with triazines. Using extracts from corn seedlings and hydroponic solutions of corn root exudates, Wenger et al. (2005) examined the reaction of DIMBOA with ATR and two of its chlorinated metabolites, DEA (2-chloro-4-amino-6-(isopropylamino)-*s*-triazine) and DIA (2-chloro-4-(ethylamino)-6-amino-*s*-triazine). DIMBOA was not detected in the corn seedling extracts following 48 h incubation nor was it detected after 11 d in the treated hydroponic solutions. However, MBOA (2,3-dihydro-6-meth-oxy-benzoxazol-2-one) was detected in the reaction solutions, and both MBOA and HMBOA (3,4-dihydro-2-hydroxy-7-meth-oxy-2H-1,4-benzoxazin-3-one) and were detected in the hydroponics solutions. The MBOA was presumed to be the result of spontaneous degradation of DIMBOA, as this is well documented (Grambow and Luckge 1986). The HMBOA in the hydroponics solutions was cited as the lactam precursor of DIMBOA in the biosynthesis pathway of plants and thus presumed to indicate DIMBOA was being synthesized by the corn roots, despite non-detection in HPLC-MS analyses.

The pH effect on the reaction between hydroxamic acid Bx compounds and triazines has been investigated by several groups (Castelfranco and Brown 1962, Tipton et al. 1971, Nakano et al. 1973). Castelfranco and Brown (1962) reported no effect on the reaction of corn extracts and simazine when pH was varied between 4-7. Tipton et al. (1971) found the reaction between DIMBOA and simazine was more rapid in mildly acidic solutions than in solutions with pH near neutral. Assuming the mechanism of

hydroxylation is driven by nucleophilic attack, this result was counter-intuitive. However, Nakano et al. (1973) found similar results when using cyprazine and model hydroxamic acids. They proposed an initial protonation reaction in which the neutral form of the hydroxamic acid protonated the weakly basic chloro-triazine. The protonation reaction simultaneously results in the formation of the anionic form of the hydroxamic acid (strong nucleophile) and enhances the susceptibility of the chloro-triazine to nucleophilic attack.

With access to more advanced spectrometric techniques and pure authentic compound, some of the inferences made decades ago can now be measured and demonstrated directly. The objective of the current research was to characterize the following reaction:

$$ATR + DIBOA\text{-}Glc \rightarrow HA + Cl^{-}(?) + Bx(?)$$

The reaction above reflects the current state of understanding based on the literature. The DIBOA-Glc-ATR reaction was characterized through a series of experiments employing a variety of chromatographic and spectroscopic techniques. The specific objectives were to quantify reactant and product kinetics and stoichiometry, identify the reaction intermediate and Bx product(s), verify the fate of Cl, and determine the influence of pH on reaction rate. In the long term, these parameters will help scientists and practitioners evaluate the potential application of DIBOA-Glc as an ATR mitigation tool, as well as contribute to our understanding of Bx chemistry and the use of natural Bx compounds for various applications.

Materials and Methods

Chemicals. Chromatography grade solvents and reagents including acetonitrile,

ammonium formate, formic acid, methanol, and concentrated phosphoric acid were purchased from Fisher Scientific (Pittsburg, PA, USA). Atrazine (98.9 %), DEA (98.2%), DIA (98.4%), DDA (97%; 2-chloro-4,6-diamino-*s*-triazine), HA (98.3%), DEHA (99.5%; 2-hydroxy-4-amino-6-isopropylamino-*s*-triazine), DIHA (>97%; 2-hydroxy-4-ethylamino-6-amino-*s*-triazine), and DDHA (98%; 2-hydroxy-4,6-diamino-*s*-triazine) were purchased from ChemService (West Chester, PA, USA). Radio-labeled ^{14}C-ATR was purchased from America Radiolabeled Chemicals, Inc. (St. Louis, MO). Sodium azide (NaN_3, 99.99+%) and BOA (98%; benzoaxazolinone-2(3H)-one) were purchased from Sigma Aldrich (St. Louis, MO). Chromatography grade water was used for sample preparation and HPLC mobile phases. Flow Logic 2:1 flow-scintillation cocktail fluid was purchased from Lab Logic Systems, Inc (Brandon, FL). DIBOA-Glc was isolated and purified from eastern gamagrass (*Tripsacum dactyloides* (L.)) as described in Willett et al. (2014).

Kinetics. An isolation method was used to determine the rate constant (k) of DIBOA-Glc and ATR degradation kinetics. In order to force pseudo-first order conditions with respect to ATR kinetics, ATR was reacted in the presence of an excess amount of DIBOA-Glc. Conversely, to isolate the kinetics of DIBOA-Glc degradation, DIBOA-Glc was reacted with excess ATR. For the ATR kinetics experiment, triplicate samples with 12 mL total volume containing 6 mL of 12 μM (1.92 μCi mL^{-1}) ^{14}C-ATR and 6 mL 1200 μM DIBOA-Glc in DI water (100:1 DIBOA-Glc:ATR) were monitored over the course of 160 hours at ambient temperature (25-28°C). Analysis of the ^{14}C-ATR was performed at 0, 1, 2, 4, 8, 16, 31, 64, 128, and 160 hrs using a Shimadzu LC-10AT VP HPLC (Columbia, MD) with a UV-detector and β-Ram flow scintillation detector (LabLogic

Systems, Inc., Brandon, FL) as described in Lin et al. (2011) and Willett et al. (2013). The mobile phase consisted of acetonitrile and aqueous 0.1% H_3PO_4 buffer with a gradient from 10%-25% acetonitrile from 0-15 min, 25-40% from 15-30 min, 40-75% acetonitrile from 30-40 min, 75-10% from 40-46 min, and 10% for 45-60 min at 1 mL min^{-1}. Flow scintillation cocktail fluid was supplied at a ratio of 2:1 to the mobile phase. During the experiment, samples were in 13-mL glass vials sealed with screw cap lids and septa and placed in the HPLC automated sampler. A 10 µL injection volume was used at each sample time; thus, the change in sample volume was negligible.

For DIBOA-Glc kinetics experiment, a NaN_3 solution was used in the sample matrix to inhibit microbial degradation of the DIBOA-Glc and ATR in solution. While inhibition of microbial growth can be accomplished many ways, the NaN_3 was chosen for several reasons. Bx compounds are known to be chelators (Tipton and Buell 1970, Raveton et al. 1997, Poschenrieder et al. 2005). While chelation is more of a concern for the aglucones, caution was taken to prevent the possibility of chelation, and Na^+ ions were thought to be less likely to chelate than polyvalent species. The kinetics of Bx reactions with triazines is influenced by the presence of solvents (Niemeyer 1988, Raveton et al. 1997), so solvent content of the samples solutions was kept to a minimum. Chefetz et al. (2006) demonstrated that while NaN_3 has been used as a biocide in ATR reaction mixtures in many sorption experiments, it can react with atrazine in solution. They found degradation was enhanced at higher concentrations (3-10 mM) of NaN_3. To avoid this, a concentration of half of their lowest concentration was selected. For DIBOA-Glc controls, 11.5 mL of 1.5 mM NaN_3 and 0.5 mL of 1200 µM DIBOA-Glc (aqueous) were combined. For ATR controls, 11.9 mL of 1.5 mM NaN_3 and 0.1 mL of

13.8 mM ATR (methanol) were combined. Triplicate treatment samples of 11.4 mL of 1.5 mM NaN_3, 0.5 mL of 1200 µM DIBOA-Glc (aqueous), and 0.1 mL of 13.8 mM ATR (methanol) (2.3:1 ATR:DIBOA-Glc) were combined. All samples remained at ambient room temperature (25-28°C) and were analyzed for DIBOA-Glc content at 0, 8, 16, 64, 128, 256, and 512 hours using HPLC analysis with UV detection as described above for the ATR kinetics experiment, but only UV detection was used. A 10 µL injection volume was used at each sample time to minimize changes in sample volume.

Following the 512 hour (21 day) sampling period, the DIBOA-Glc kinetics samples were put into cold storage at 0ºC and stored for analyzsis using LC/MS/MS to determine reaction products formed. Data from the kinetics studies were graphed as µmol L^{-1} versus seconds. Function coefficients and r^2 were determined by non-linear regression using SigmaPlot 12.5 (Systat Software, Inc., San Jose, CA).

Intermediate Identification. A reaction mixture of DIBOA-Glc and ATR was prepared for LC/MS/MS analysis to identify a potential reaction intermediate observed during the ATR kinetics experiment. The treatment samples consisted of 850 µL of 2000 µM DIBOA-Glc and 850 µL 500 µM ATR in 34% methanol solution. For the controls, 850 µL of 2000 µM DIBOA-Glc were combined with 850 µL of 50% methanol solution for a final methanol content of 34%. Treatment and control samples were prepared in triplicate. A single ATR-only control (35% methanol) was also made, containing 850 µL of 20% methanol solution and 850 µL of 500 µM ATR. Analyses were carried out at the University of Missouri Mass Spectrometry Facility on a Finnigan Ion trap (San Jose, CA) with electrospray ionization (ESI) in positive (+) mode. The method consisted of a 10 µL injection volume on a Luna C_{18} (2) 150 x 2.0 mm, 5µ column (Phenomenex, Torrance,

CA) with an oven temperature of 40ºC, and 0.1% formic acid and acetonitrile as the mobile phases. The acetonitrile was ramped from 1% to 99% over 15 min, held at 99% for 2 min, then brought back to 1% for 3 min. Total flow was 0.3 mL min^{-1}.

Chloride Formation. Samples containing 2.5 mL of 2000 µM DIBOA-Glc and 2.5 mL of 40 µM ATR (50:1 DIBOA-Glc:ATR) with methanol content less than 2%, were incubated at room temperature for >160 hours to ensure completion of the reaction. Atrazine only, DIBOA-Glc only, and water blanks were tested concurrently. Samples, controls, and blanks were analyzed for Cl^- concentration using a Dionex ICS-1000 ion chromatography system as described in Gaddie (2012). The ion chromatography (IC) system was equipped with an IonPac® AG14A 4 mm guard column (4×50 mm) and an IonPac® AS14A analytical column (4×250 mm) and AS40 auto-sampler system (Dionex Corp., Sunnydale, CA). Dionex AS14A eluent (8.0 mM sodium carbonate; 1.0 mM sodium bicarbonate in ultrapure water) at a flow rate of 1 mL min^{-1} was used for anion anaylsis with the mobile phase; the injection volume was 20 µL. Samples were corrected for background Cl^- using the average concentration of 5 blanks. Data were analyzed by a one-way ANOVA and tested for significant differences using the Tukey Test (p=0.05) for differences among treatment means in SigmaPlot 12.5.

pH Effects. DIBOA-Glc and ATR were reacted in 0.03 ± 0.002 M $NaNO_3$ solution adjusted with HNO_3 and NaOH to achieve values ranging from pH 4 to 9. Solution pH was measured using an Amani 1000 microminature pH probe (Innovative Instruments, Inc. Tampa, FL) with an Orion Star A 111 benchtop pH meter (ThermoScientific, Waltham, MA). For each pH treatment, 2 mL samples contained 10 µL of 20,000 µM DIBOA-Glc, 10 µL of 2,000 µM ATR, 1890 µL of the $NaNO_3$ solution, and 2.5%

methanol (associated with atrazine addition); the molar ratio of DIBOA-GLC to ATR was 100:1. Atrazine controls consisted of 10 µL of 2,000 µM ATR (50% methanol/50% water), 100 µL of DI water, and 1890 µL of $NaNO_3$ solution. Blanks contained 2 mL of the $NaNO_3$ solution matrix. Samples, controls, and blanks were prepared in triplicate and left to react for 16 hours at ambient room temperature. Following the reaction period, each reaction vessel was subjected to liquid/liquid extraction with dichloromethane (2 mL x 3). The organic fraction was collected, evaporated to dryness under N_2 (g) at 40 ºC, and resuspended in 1 mL of 40% acetonitrile/60% water. Atrazine concentration was quantified using a Shimadzu LC-10AT VP (Columbia, MD) HPLC equipped with a UV detector with a 20 min isocratic method on a Columbus C_8 column (250x4.60 mm, 5 µm; Phenomenex, Torrance, CA). The mobile phase consisted of 55% solvent A (0.1% H_3PO_4 aqueous buffer) and 45% solvent B (acetonitrile) at a flow rate of 1 ml min^{-1}. Ultraviolet absorbance was monitored at 220 nm. Results were analyzed with a one-way ANOVA and significant differences were determined by Tukey's method (p=0.05) in SigmaPlot 12.5.

Bx Product identification. The samples from the DIBOA-Glc kinetics study were used to identify the Bx reaction product(s). Analyses were carried out by the University of Missouri Mass Spectrometry Facility on a Waters (Milford, MA) H-Class UPLC system with quaternary solvent manager and auto sampler with a Xeno TQ-S tandem quadrupole mass spectrometer. The injection volume was 10 µL, and the column was a 2.1x50 mm Acquity UPLC BEH C_{18} (1.7µm) heated to 40º C. The mass scan range was 50-550. In addition to MS/MS data, the samples were scanned for UV absorption over a range of 190-500 nm with an in-line photodiode array detector (PDA). A 6 min method with a

three component mobile phase of 0.1% formic acid in acetonitrile, methanol, and 10 mM ammonium formate was used at a flow rate of 0.4 mL min^{-1}. The initial conditions were 2% methanol and 98% ammonium formate. Methanol was then linearly increased to 100% over 4 min, and then held for 0.3 min. At 4.4 min, the mobile phase was switched to 98% formic acid and 2% methanol. This condition was held for 1 min. At 5.5 min, the mobile phase composition was 2% methanol and 98% ammonium formate. This was held for 0.5 min to flush the column.

Results and Discussion

Kinetics. In the presence of excess ATR, DIBOA-Glc disappearance followed 1st order degradation kinetics (r^2= 0.998) with a rate constant (k) of 1.43 x 10^{-6} sec^{-1} ± 1.14 x 10^{-7} (standard error, SE), and demonstrated that the phytochemical was being consumed ($t_{1/2}$ = 134.6 h) in the reaction and not acting catalytically (Figure 4.3). There has been some

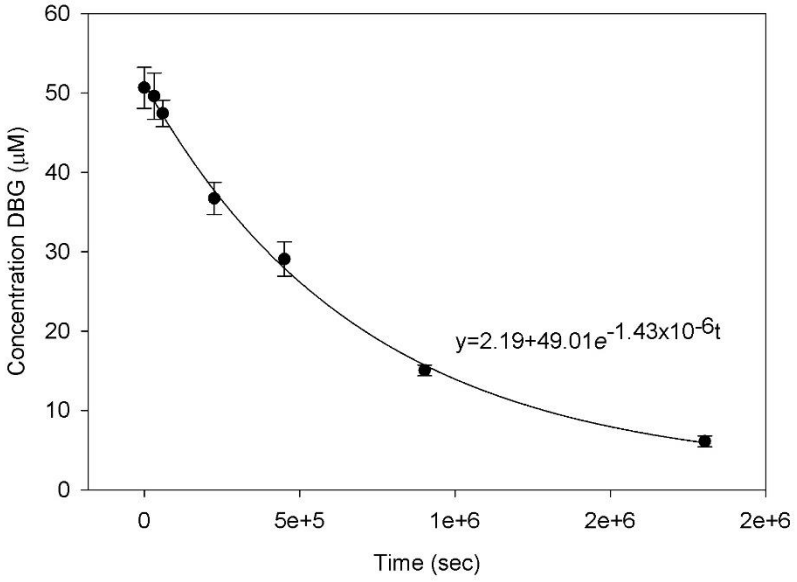

Figure 4.3 Degradation of DIBOA-Glc in the reaction of 50 µM DIBOA-Glc and 115 µM ATR over 512 hours. Error bars represent standard deviation from the mean. DIBOA-Glc fits a 1st order degradation kinetics model (r^2=0.998). The disappearance of DIBOA-Glc demonstrated that it was consumed and was not a catalyst in the reaction.

debate in the literature regarding the possibility of a catalytic reaction between Bx species and triazine herbicides (Hamilton and Moreland 1962, Tipton et al. 1971, Nakano et al. 1973, Niemeyer 1988, Raveton et al. 1997, Niemeyer 2009). Early investigators believed that the Bx compound remained intact in the reaction because they could not detect degradation products using UV spectrophotometry and TLC (Hamilton and Moreland 1962, Tipton et al. 1971), and the notion that Bx compounds were catalysts became accepted in the literature (Nakano et al. 1973, Niemeyer 1988). Raveton et al. (1997) inferred that the catalytic hypothesis was not valid based on their findings of 1^{st}-order degradation of ATR to HA, and a new mechanism was proposed. However, these results were not considered definitive as the Bx compound, DIMBOA, was not directly measured nor was the proposed chlorinated Bx product. In the comprehensive and widely cited review of Bx compounds by Niemeyer (2009), it was noted that the question of a catalytic role of Bx compounds in degrading triazines remains unresolved. The data presented here clearly shows that the Bx reactant, DIBOA-Glc, was consumed in the reaction with atrazine, and in no way supports a catalytic mechanism hypothesis for the reactants studied.

The degradation of ATR in the presence of excess DIBOA-Glc also exhibited 1^{st}-order kinetics (r^2= 0.9997) with a $k = 1.17 \times 10^{-5}$ $sec^{-1} \pm 2.34 \times 10^{-7}$ (SE). The half-life of ATR was 20 h. As consistent with previous studies, the major degradation product of ATR was HA (Castelfranco et al. 1961, Castelfranco and Brown 1962, Hamilton and Moreland 1962, Tipton et al. 1971, Raveton et al. 1997). The degradation of ATR and formation of HA by DIBOA-Glc over time can be seen in Figure 4.4. The formation of

HA also demonstrated 1st-order kinetics and a 1:1 stoichiometric conversion of ATR to HA; 5.5 ± 0.19 µM ATR converted to 5.3 ± 0.10 µM of HA after 160 hours of reaction.

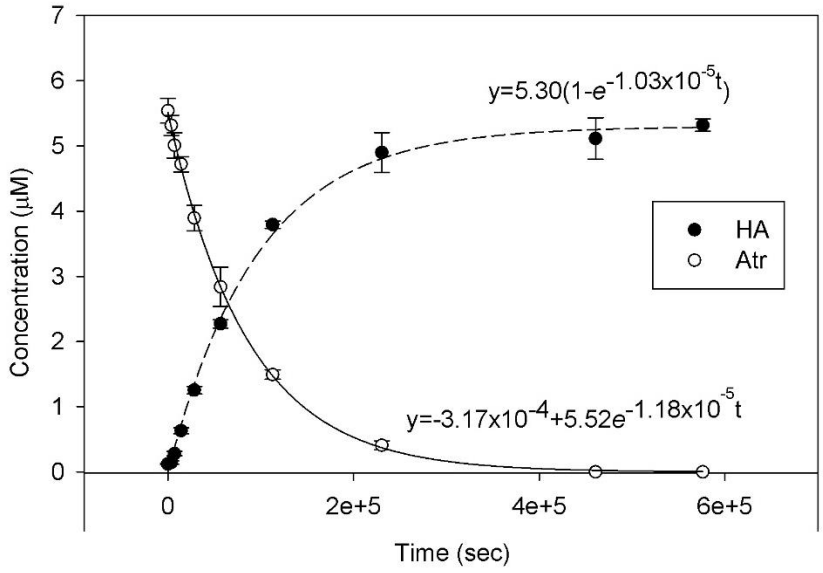

Figure 4.4 Formation of hydroxyatrazine (HA) and degradation of atrazine (ATR) in the presence of excess DIBOA-Glc. Error bars represent standard deviation from the mean. Atrazine disappearance fit a 1st order degradation kinetics model (r^2>0.999), while HA formation fit a 1st order exponential rise to a maximum model (r^2=0.998).

Intermediate Identification. During the ATR kinetics study, an unknown ^{14}C-labeled compound was observed within the first 4 hours of the reaction followed by slow dissipation over the next 28 h (Figure 4.5). The retention time (R_t = 28.5 min) of the compound did not match any of seven ATR metabolite standards nor that of the ATR and DIBOA-Glc standards. However, the detection of a radiolabeled compound indicated it was derived from the ^{14}C-ATR used in the experiment.

The results of the MS/MS analysis of reaction solutions containing DIBOA-Glc + (unlabeled) ATR revealed a pseudomolecular ion, $[M+H]^+$, of m/z 523 with product ion spectra containing the following ions (m/z) and relative abundances (%): 361 (100%, $[M-162]^+$); 416 (25%, $[M-107]^+$); 198 (6.1%, $[M-325]^+$), and 343 (4.2%, $[M-180]^+$) (Figure

4.6). Two of the product ions were derived from DIBOA-Glc, including m/z 361, indicating the loss of glucose less a water molecule from DIBOA-Glc, and m/z 343,

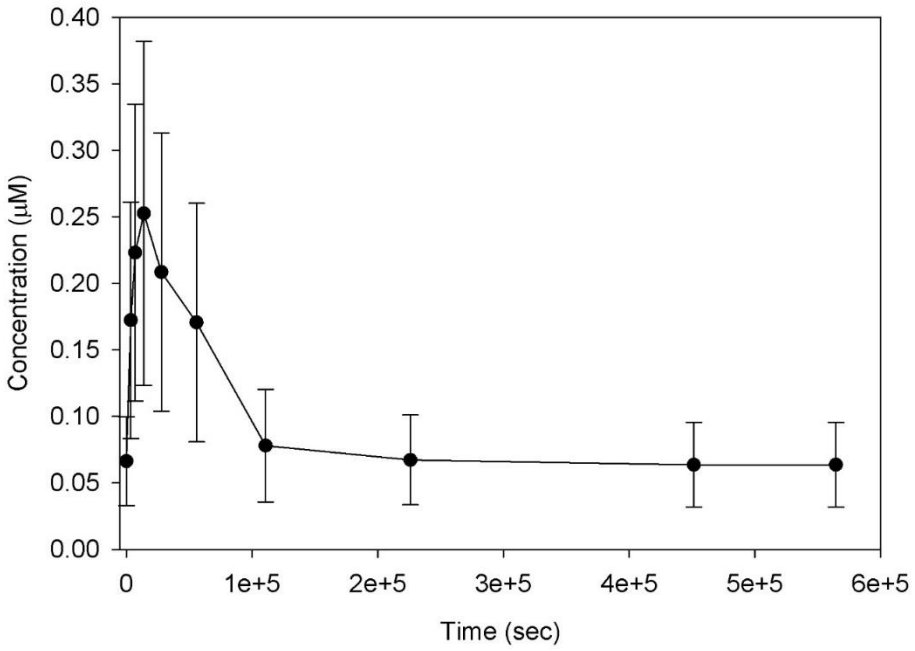

Figure 4.5 Reaction intermediate formation and degradation over time in a reaction mixture of 6 µM ^{14}C-atrazine and 600 µM DIBOA-Glc. Error bars represent the standard deviation from the mean.

representing the loss of glucose. This fragmentation pattern is common to glucosylated Bx compounds (Willett et al., 2013). The fragment ion m/z 198 is the protonated HA species, which arises from the loss of DIBOA-Glc less a water from the parent ion [M-325]$^+$. The loss of 107 from the parent ion to give m/z 416 was tentatively identified as [M - C_6H_5NO]$^+$. The pseudomolecular ion and the product ion spectra supported the formation of a conjugate structure composed of DIBOA-Glc and HA (Fig. 4.6). A reaction intermediate was proposed by Castelfranco and Brown (1962) and Nakano et al. (1973), but the existence of the intermediate was not demonstrated using naturally

occurring Bx compounds, such as DIBOA-Glc. Results presented here support the conjugate structure proposed by Nakano et al. (1973) in which the two reactants bind via

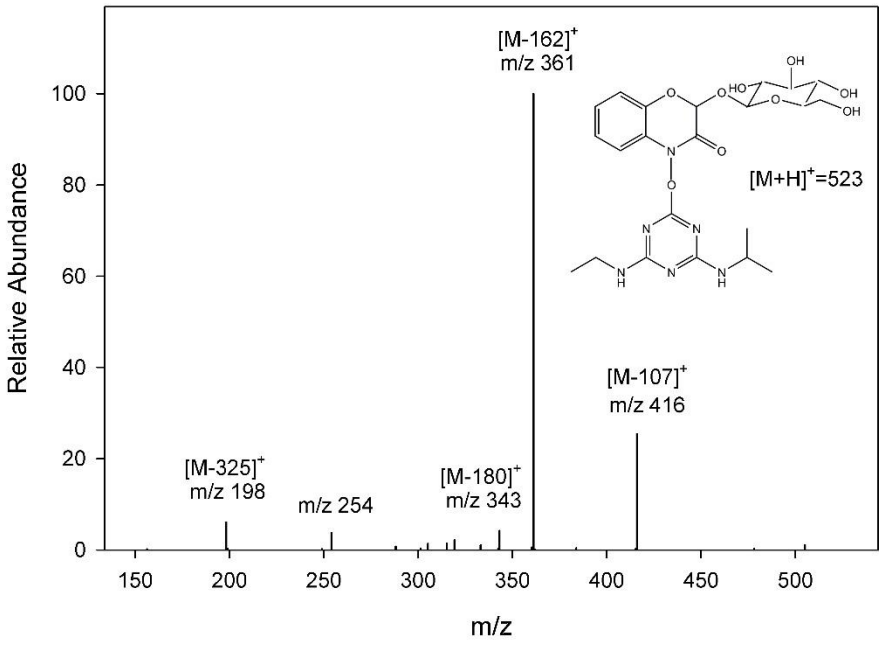

Figure 4.6. Tentative identification of pseudo-stable reaction intermediate as a hydroxyatrazine/DIBOA-Glc conjugate with a shared O at the C-2 position and hydroxamic acid-N, respectively. MS/MS spectra showed $[M+H]^+$ 523 at a retention time of 10 min on UPLC. The major product ion m/z 361 represents the loss of glucose less a water $[M - C_6H_{12}O_6 + H_2O]$ from the m/z 523 species.

the hydroxamic acid moiety with the release of Cl^- into solution. Although the concentration of the reactants and products was not quantified over the course of the reaction, the conjugate structure of the intermediate provided direct evidence of a stoichiometric ratio of 1:1 DIBOA-Glc:ATR for the reaction, and definitely confirmed the reaction mechanism to be nucleophilic attack by the hydroxamic acid at the C-2 position of the triazine ring.

Chloride formation. Chloride formation via the DIBOA-Glc-ATR reaction was observed with an average of 21.4 ± 8.45 μM Cl^- in the DIBOA-Glc + ATR samples, compared to

6.48 μM ± 2.02 and 0.00 μM Cl^- in the DIBOA-Glc-only and ATR-only controls, respectively. The reaction samples contained a significantly greater amount of Cl^- than the controls and therefore it was concluded that Cl^- is indeed a product of the reaction. Further, the Cl^- formed was within analytical error of the expected 1:1 molar ratio of atrazine consumed and Cl^- formed. This confirms the inference suggested in Castelfranco et al. (1961) in which the formation of Cl^- was hypothesized (Figure 4.1), but until this research has never been demonstrated and quantified. Moreover, these results bring resolution to the fate of Cl in chlorotriazine-Bx reactions and refute previous speculation regarding the formation of chlorinated Bx compounds (Raveton et al., 1997) (Figure 4.2).

pH Effects. Data from the variable pH experiments showed greater reactivity of DIBOA-Glc under acidic conditions compared to neutral or basic pH (Figure 4.7). Reactivity at pH 4 was significantly greater than all higher pH treatments. With a calculated pK_a= 7.6 (SciFinder; Chemical Abstracts Service) for DIBOA-Glc, this finding was seemingly inconsistent with the reaction mechanism which proposes that DIBOA-Glc acts as a nucleophile. Intuitively, a more basic solution should favor a more negatively charged species, and therefore increase the nucleophilicity of the DIBOA-Glc. However, these results were consistent with past findings in which the triazine-Bx reaction rate was greater at low pH (Tipton et al. 1971, Nakano et al. 1973, Raveton et al. 1997). Nakano et al. (1973) discuss the various structural forms of the hydroxamic acid moiety as a function of pH. They found that the neutral form of N-hydroxysuccinimade was 5 times more nucleophilic towards cyprazine than its base (anionic) form. They proposed that an initial protonation reaction results in the anionic and highly nucleophilic form of the Bx compound and a protonated and therefore more electrophilic triazine molecule. Results

presented here would support this proposed reaction mechanism for the enhanced reaction rate under acidic solution conditions.

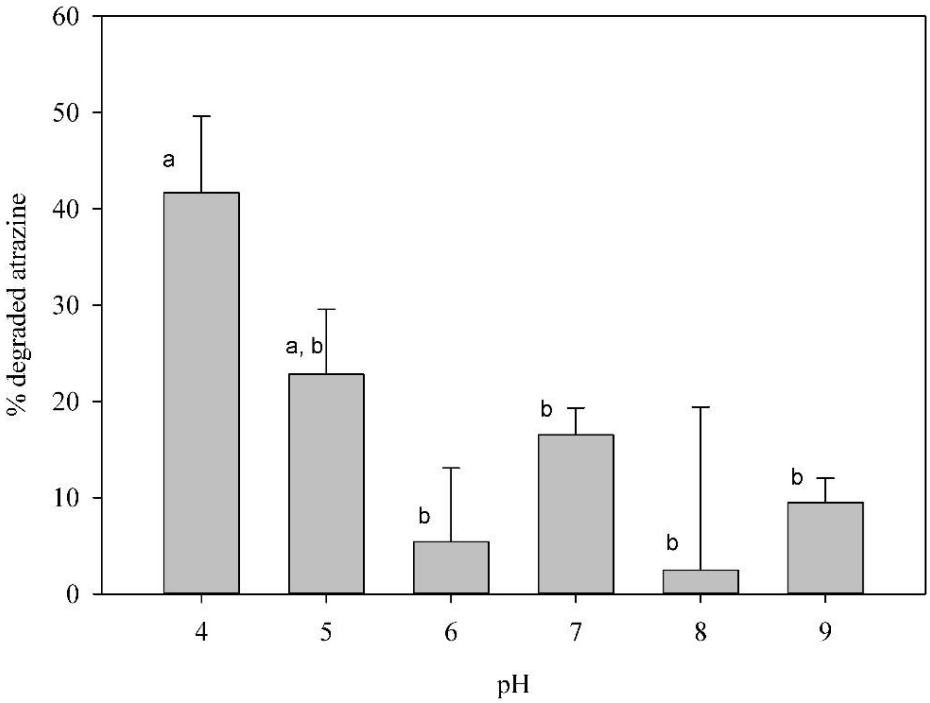

Figure 4.*7*. Effect of solution pH on the reaction between 100 µM DIBOA-Glc and 10 µM atrazine measured after 16 hours. Solution pH was achieved by addition of HNO_3 and/or NaOH to a $NaNO_3$ electrolyte matrix with I= 0.03 ± 0.002 M. Bars with different letters indicate significant differences ($p<0.05$). Error bars represent standard deviation.

Bx Product Identification. Samples from the DIBOA-Glc kinetics experiment were analyzed for the presence of Bx products initially by HPLC/UV and then by UPLC/MS/MS. Only one Bx compound was identified in the reaction mixtures of DIBOA-Glc and ATR. In the HPLC/UV chromatograms, this peak eluted at R_t= 14.5 min, a time greater than DIA (R_t=12.5 min) and less than DEA (R_t=18.5 min). Analysis by UPLC/MS/MS also showed a peak with retention time between that of DIA (R_t=2.29 min) and DEA (R_t=2.76 min) that did not correspond with retention times of DIBOA-Glc, ATR, or ATR metabolite standards. With a pseudo-molecular ion $[M+H]^+$ of m/z

136, the peak was identified benzoxazolin-2(3H)-one (BOA). The peak matched a BOA standard based on four points of identification: retention time, pseudomolecular ion mass, MS/MS product ion spectra (Figure 4.8), and PDA spectra. Retention time was 2.55 min, with λ_{max} at 196, 226, and 271. The m/z 65 was the dominant fragment, with relative abundance of 100% for both the BOA standard and the reaction mixture. Relative abundance of all other fragment ions also closely matched (Figure 4.8).

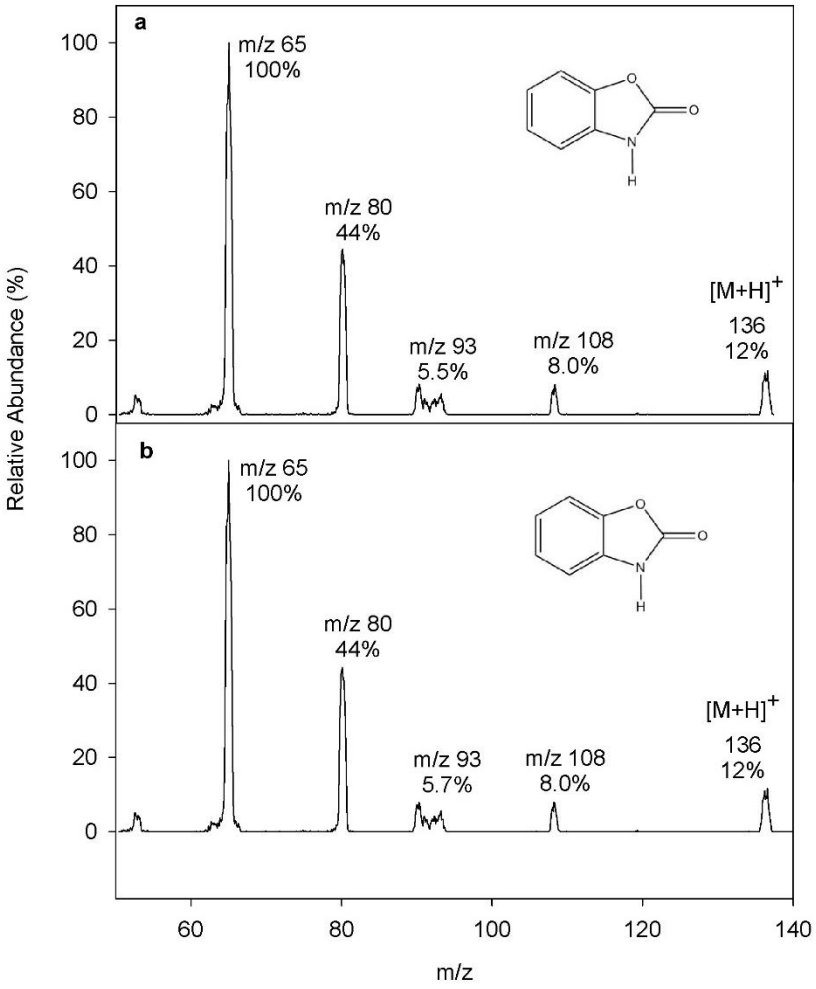

Figure 4.8. MS/MS spectra for a) BOA standard and b) unknown peak occurring at R_t= 2.55 min in reaction mixtures of DIBOA-Glc and atrazine. The MS/MS for the unknown peak is nearly an exact match to that of the authentic BOA standard. The identity of the 2.55 min peak was additionally confirmed with retention time and UV spectra that matched the BOA standard.

While the identity of BOA in the reaction mixtures was highly certain, its presence does not provide clarity to the direct fate of DIBOA-Glc in the reaction. There were 11.9 +/- 0.58 (SD) µM BOA in the reaction mixture samples tested. This accounts for < 25% of the DIBOA-Glc that was originally added to the mixture, of which only 6.0 ± 0.66 µM remained as parent compound at the time of the last injection on HPLC (512 hours). While the samples were placed in cold storage before the MS/MS analysis, 2 months elapsed between the 512 hour injection on HPLC and the injection on LC/MS/MS. It is possible that the samples could have continued to undergo reactions prior to the LC/MS/MS final analysis. This leaves a possibility that the BOA was present in greater quantities and may have degraded prior to the quantitation by MS/MS, or may indicate microbial degradation of the reaction products.

Proposed Reaction Pathway. The mechanism for which the BOA may have arisen from the reaction was not clear, but there appears to be two possibilities. One would be a spontaneous degradation of DIBOA-Glc to BOA in a manner analogous to that reported for the aglucones, DIBOA and DIMBOA, to their respective bezoxazolinones, BOA and MBOA (Bravo and Niemeyer 1986, Niemeyer 1988). However, such a mechanism for the glucosylated species has not been reported to date and cannot account for the mechanism by which deglycosylation occurred. The second possibility was microbial contamination of the samples. Under the mild conditions employed in the DIBOA-Glc kinetic experiment, enzymatic cleavage of the glucose moiety was a possibility despite efforts to suppress microbial growth with NaN_3. Deglycosylation of DIBOA-Glc to DIBOA would then allow for subsequent spontaneous degradation to BOA, and given the length of the initial kinetic experiment and subsequent storage time before MS/MS

analyses were performed, this was the most likely possibility for the presence of BOA and the absence of other intermediate Bx compounds. Furthermore, the conversion of hydroxamic acids to benzoxazolinones is known to be incomplete (Niemeyer 1988), and the fact that the BOA only accounts for 25% of the added DIBOA-Glc was consistent with this finding.

A possible insight into the presence of BOA and lack of other Bx reaction products may be found in Wenger et al. (2005). When hydroponic solutions of corn exudates were reacted with atrazine and then analyzed for Bx compounds, the expected presence of DIMBOA was not detected. Instead, they found the lactam HMBOA and benzoxazolinone MBOA. While they concluded HMBOA was present as a biosynthetic precursor to DIMBOA, we speculate that it was the product of the DIMBOA-ATR degradation reaction. If the glucoside was cleaved from DIMBOA-Glc via microbial degradation, the degradation of DIMBOA to BOA would have occurred spontaneously. If HBOA-Glc formed, it would have likely been subject to degradation via a similar microbe-mediated pathway.

While the presence of BOA did not provide useful information about the fate of DIBOA-Glc or the reaction mechanism, combining the current findings with those reported in the literature, a two-step reaction mechanism is proposed (Figure 4.9). The reaction between DIBOA-Glc and ATR is the result of a nucleophilic attack occurring between the nucleophilic –NOH (δ^-) of the Bx species, DIBOA-Glc, and the electrophilic center (δ^+) at the C-2 position of the triazine species, ATR. In the first step, a reaction intermediate, a conjugate of DIBOA-Glc and ATR, is formed and Cl^- is released into solution. The second step involves dissociation of the conjugate to form HA and the

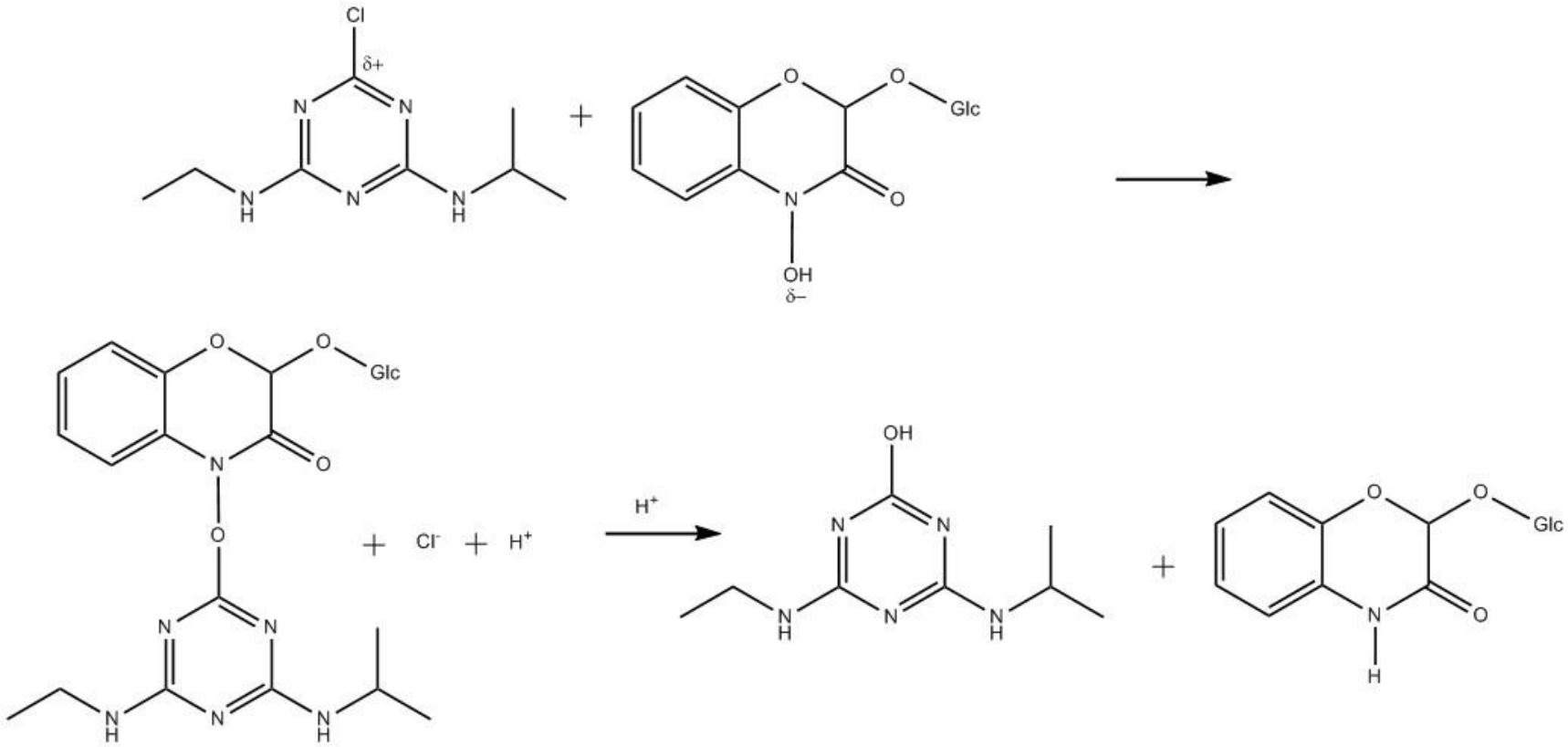

Figure 4.9 Proposed reaction mechanism for the degradation reaction between DIBOA-Glc and atrazine, involving a nucleophilic attack by the –NOH of the DIBOA-Glc at the C-2 position of the atrazine. This initially results in formation of a conjugate intermediate and release of Cl^-. Upon disintegration of the conjugate, hydroxyatrazine and HBOA-Glc are formed. DIBOA-Glc is not regenerated.

lactam HBOA-Glc. The reaction follows 1st order kinetics in terms of ATR and DIBOA-Glc degradation and HA formation, and is 2nd order overall with stoichiometry of 1:1 DIBOA-Glc:ATR. The formation of a conjugate reaction intermediate was identified by LC/MS/MS, and further supports the 1:1:1 reaction stoichiometry of DIBOA-Glc:ATR:HA. This is in contrast to an early speculation put forth by Tipton et al. (1971), who suggested a molecular aggregate of the Bx compound was reacting with simazine to form hydroxysimazine. This was likened to the multi-layered structure of montmorillinite clay that facilitates the hydroxylation of simazine. However, the data here does not support such a hypothesis, and clearly demonstrates a 1:1 stoichiometry. The proposed mechanism shows the formation of free Cl^- which was indicated in the results of the IC analysis, and is in agreement with the mechanism hypothesized by Castelfranco et al. (1961) nearly 50 years ago. Informed by the results of the DIBOA-Glc kinetics showing degradation of the Bx compound in the reaction with ATR, the DIBOA-Glc is consumed in the reaction and is not regenerated upon separation of the conjugate into HA and a Bx product. Lastly, the mechanism proposes that the DIBOA-Glc is degraded into HBOA-Glc. The lack of evidence showing HBOA-Glc in this research is likely a result of a weakness in the experimental design and may not fully capture the realities of the Bx product formation. However, the formation of HBOA-Glc was supported by the fact that HA contains the oxygen atom derived from the hydroxamic acid of DIBOA-Glc and the observed HMBOA in the reaction mixtures reported by Wenger et al. (2005). In this study, the reaction mixtures were stored for a period of time which likely allowed microbial degradation to alter the composition of the solutions. In the future, alternative measures should be taken to prevent microbial contamination. Additionally, it is

suggested that the reaction be monitored over the course of the entire reaction period using LC/MS/MS rather than being analyzed singularly at the end of the reaction.

Conclusion

Through this research, the state of knowledge concerning the reaction between ATR and DIBOA-Glc has been advanced. Several concepts that were speculated on in early research were for the first time demonstrated here by original data using modern chromatography. Four aspects of the Bx-ATR reaction were confirmed: 1) formation of Cl^- as first proposed by Castelfranco et al. (1961); 2) the formation and degradation of a reaction intermediate with a conjugate structure composed of HA and DIBOA-Glc, first put forth by Nakano et al. (1973); 3) definitive evidence for a non-catalytic role of the Bx reactant as seen by the loss of DIBOA-Glc in the presence of excess ATR; and 4) data supporting a 1:1 stoichiometry between reactants and products. This work has reproduced the pH effect on the reaction between DIBOA-Glc and ATR that others have observed using similar compounds such as simazine, cyprazine, DIMBOA-Glc, and other model hydroxamic acids (Nakano et al. 1973, Raveton et al. 1997). The kinetics for the reaction between DIBOA-Glc and ATR are reported here for the first time. Although the identity of the DIBOA-Glc product was not definitively demonstrated, a reaction mechanism was proposed that includes HBOA-Glc as the Bx product and offers an alternate hypothesis to explain the findings of Wenger et al. (2005).

These reaction parameters have important application for evaluating DIBOA-Glc as a potential ATR mitigation tool. For example, knowing that the reaction is enhanced by low pH has implications for use of the phytochemical in alkaline soils. Or, the fact that DIBOA-Glc is consumed in the reaction rather than playing a catalytic role indicates that

it has less potential to be persistent in the environment and would require seasonal applications to address the problems associated with seasonal ATR application. When DIBOA-Glc is the limiting factor, the reaction takes 512+ hours to complete, and this is under ideal conditions. The relatively slow kinetics of the reaction suggest that it would take several weeks for the DIBOA-Glc to react fully with the ATR it would encounter in the soil environment, implying that this technology would likely be best suited for use in combination with other mitigation strategies such as vegetative buffer strips. While this research begins to cast light on questions of practical application, it is clear there is much left to learn about the potential of DIBOA-Glc and other Bx compounds for use as natural products for herbicide remediation.

CHAPTER 5: CONCLUSION

Through the course of the dissertation research the three study objectives described in Chapter 1 were accomplished.

Study 1, presented in Chapter 2, reported results identifying and quantifying Bx (2-hydroxy-2*H*-1,4-benzoxazin-3(4H)-one) compounds in eastern gamagrass (*Tripsacum* dactyloides (L.) L., EG) roots. The two Bx compounds identified were HBOA-Glc (2-β-D-glucopyransosyloxy-1,4-benzoxazin-3-one) and DIBOA-Glc (2-β-D-glucopyransosyloxy-4-hydroxy-1,4-benzoxazin-3-one). A major finding of Study 1 was identifying DIBOA-Glc as the atrazine (6-chloro-*N*-ethyl-*N*'-(1-methylethyl)-1,3,5-triazine-2,4-diamine)-degrading compound present in EG roots. This work provides the first peer-reviewed evidence of the occurrence of Bx compounds in the *Tripsacum* genus.

Through **Study 2**, detailed in Chapter 3, methods for the isolation and purification of DIBOA-Glc directly from methanol extractions of EG roots were developed. The method is superior to other published methods in that it uses non-hazardous chemicals on standard HPLC equipment that can be readily automated. Whereas currently published methods require handling hazardous chemicals, expertise in synthetic chemistry, or use of non-standard lab equipment, the method developed in Study 2 will allow a greater number of researchers to produce bulk quantities of highly pure DIBOA-Glc for future work, and the method could be readily adapted for purification of related Bx compounds in a variety of plant species.

Lastly, **Study 3**, described in Chapter 4, provides a characterization of the ATR/DIBOA-Glc degradation reaction in terms of kinetics, intermediate and product compound formation, stoichiometry of the degradation reaction, and pH effects. The ATR/DIBOA-Glc degradation reaction was found to follow 1^{st} order kinetics in terms of the reactants, as well as the formation of HA (2-hydroxy-4-ethylamino-6-isopropylamino-*s*-trazine). The results of the kinetics experiments demonstrated that DIBOA-Glc is consumed in the one-way degradation reaction with ATR and does not act as a catalyst. The structure of a reaction intermediate, which had previously been postulated by others, was for the first time characterized with MS/MS data for the naturally occurring Bx compound DIBOA-Glc. The reaction intermediate is a conjugate between DIBOA-Glc and HA with $[M+H]^+$ of m/z 523. HA, Cl^-, and BOA (benzoxazolin-2(3H)-one) were all identified as reaction products. The formation of HA as a product was consistent with previous findings, but this work was the first to demonstrate formation of Cl^- as a product. Results from MS/MS analyses of reaction mixtures of DIBOA-Glc and ATR clearly showed the presence of BOA. However, the presence of BOA was likely an indication that the NaN_3 concentration used was not sufficient for suppressing microbial degradation over the course of the analyses. Despite the lack of direct evidence of the product of DIBOA-Glc, a new reaction mechanism was proposed in which DIBOA-Glc is degraded to the lactam, HBOA-Glc, in a noncatalytic reaction with ATR. Future studies should use additional means to eliminate microbial degradation of the reaction products and should be monitored with LC/MS/MS throughout the course of the reaction.

The collective work of the dissertation research provides meaningful contributions to Bx and natural product research. However, the research has brought to light the need for future work in the area. The current findings have advanced the understanding of the DIBOA-Glc/ATR degradation reaction, but many questions still remain. Determination of the DIBOA-Glc product formed immediately following formation of the DIBOA-Glc/HA conjugate is of primary importance. Additionally, while ATR was the main focus of the research, an investigation of DIBOA-Glc reactivity toward other pesticides is warranted. The mechanism of nucleophilic attack is sufficiently non-specific that DIBOA-Glc or other Bx hydroxamic acids could possibly react with a nucleophilic center on other organic contaminants, thereby, enhancing degradation. Non-triazine pesticides that are frequent contaminants of soil and water resources would be most worthwhile to investigate (e.g., acetanilides, isoxazoles, glyphosate). The list could be extended to include emerging contaminants of environmental concern such as veterinary antibiotics. Soil dissipation studies are needed to advance the understanding of DIBOA-Glc behavior in the soil environment. An attempt was made to determine the effect of soil dissipation on the DIBOA-Glc/ATR reaction, but useful results were not obtained due to lack of methods for proper soil extraction and separation of the reactive components. Simple solvent extraction of soil may effectively remove the DIBOA-Glc and the ATR, but they will continue to react in the extracted solution. Ideally, a simple method that interrupts the reaction and allows quantification of either ATR or DIBOA-Glc through time should be developed. This may involve the use of pH changes or chelators to de-activate the DIBOA-Glc while it or the ATR are removed from the soil. Given that soil dissipation is fundamental to understanding the behavior of the DIBOA-

Glc/ATR reaction in a real world setting these types of developments are of critical importance to advancing the work of assessing the utility of DIBOA-Glc as an ATR mitigation tool. It is hoped that this line of research will continue and will facilitate a greater effort to create more sustainable food, fuel, and fiber production practices that eliminate or reduce environmental impacts to water resources.

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VITA

Cammy Drost Willett, (born Cammy Christine Drost), was born in Kittanning, Pennsylvania on March 15, 1985 to parents Paul and Christine Drost. The family, including three older sisters, Kendra, Megan, and Lindsay, moved to Abingdon, MD in 1991. The early years living in the Chesapeake Bay area, with its rich ecology and emerging environmental concerns, were formative in the developed of her fervent interest the natural world. In 1998, she and her parents moved to Springfield, Missouri, where she completed her high school degree, graduating from Hillcrest High School as valedictorian in 2003. From there, she earned a B.S. degree in biology from Evangel University, and graduated summa cum laude in 2007. In the summer of 2007, she moved to Columbia, Missouri to pursue a master's degree in soil science at the University of Missouri under Drs. Robert Lerch and Stephen Anderson. Some of her most fond memories of graduate school were formed during the hours spent collecting data on the streambanks of NE Missouri. In 2009, she married the love of her life and fellow MU grad, Peter Willett of Columbia, Missouri (BS '07, MEd '09). She completed her MS in 2010 and plunged immediately into a PhD program, also in soil science. In 2012-1013, while working on her PhD, she was employed by the USDA Agricultural Research Service as a lab

technician in the Water Quality and Cropping Systems Unit. In of the summer of 2014, she began working for the University of Missouri in the Soil, Environmental and Atmospheric Science department as an advisor and instructor for the Introduction to Soils laboratory. She received her PhD in soil science, under Drs. Robert Lerch and Keith Goyne, from the University of Missouri in December 2014.