JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Identification of an Atrazine-Degrading Benzoxazinoid in Eastern Gamagrass (*Tripsacum dactyloides*)

Cammy D. Willett,*[,†] Robert N. Lerch,[‡] Chung-Ho Lin,[#] Keith W. Goyne,[†] Nathan D. Leigh,[⊥] and Craig A. Roberts[∥]

[†]Department of Soil, Environmental and Atmospheric Sciences, 302 ABNR Building, University of Missouri, Columbia, Missouri 65211, United States

[‡]Cropping Systems and Water Quality Research Unit, Agricultural Research Service, U.S. Department of Agriculture, 269 Agricultural Engineering Building, Columbia, Missouri 65211, United States

[#]Department of Forestry, Center for Agroforestry, 203 ABNR Building, University of Missouri, Columbia, Missouri 65211, United States

[⊥]Department of Chemistry, 125 Chemistry Building, University of Missouri, Columbia, Missouri 65211, United States

[∥]Division of Plant Sciences, 214D Waters Hall, University of Missouri, Columbia, Missouri 65211, United States

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 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.

KEYWORDS: *DIBOA-Glc, HBOA-Glc, atrazine degradation, benzoxazinone, Eastern gamagrass, Tripsacum dactyloides*

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.[1−6] Research has aided in the development of agricultural practices for mitigating and preventing atrazine contamination of water resources, such as herbicide incorporation,[7,8] reduced application rates, and establishment of vegetative buffers.[9−11] Despite intensive efforts to mitigate atrazine transport over the past 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 water and groundwater supplies.[2,12]

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.[13−16] These compounds are known to be allelopathic and to impart resistance to fungi, bacteria, and insects.[15,17−20] Due to the allelopathic nature of these compounds, use of these biomolecules as natural herbicides has been investigated.[21] Interestingly, it has also long been known that Bx compounds impart resistance to triazine herbicides such as atrazine and simazine.[22,23] 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.[10,11] Lin et al.[11] 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 day incubation trial, the most of the seven grass and forage species tested. In addition, Lin et al.[11] 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.[10,11] Yang[24] also reported that the

Received: February 14, 2013
Revised: July 24, 2013
Accepted: July 25, 2013
Published: July 25, 2013

dx.doi.org/10.1021/jf402271h | J. Agric. Food Chem. 2013, 61, 8026−8033

degradation of the triazine explosive 1,3,5-trinitroperhydro-1,3,5-triazine (RDX) was enhanced in the rhizosphere soil of EG plants.

On the basis of 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,[13−15] 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, on the basis of 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 1980s.[13−15] 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, USA). Radiolabeled ^{14}C-atrazine was purchased from America Radiolabeled Chemicals, Inc. (St. Louis, MO, USA). 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, MO, USA. 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 −4 °C, and the leaves and stems were discarded. Sample preparation procedures following root collection are outlined in Figure 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 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 35−40 °C with a stream of N_2 to ∼10 mL. A 500 μL aliquot was removed from each sample, evaporated to dryness, and resuspended in 1 mL of water.

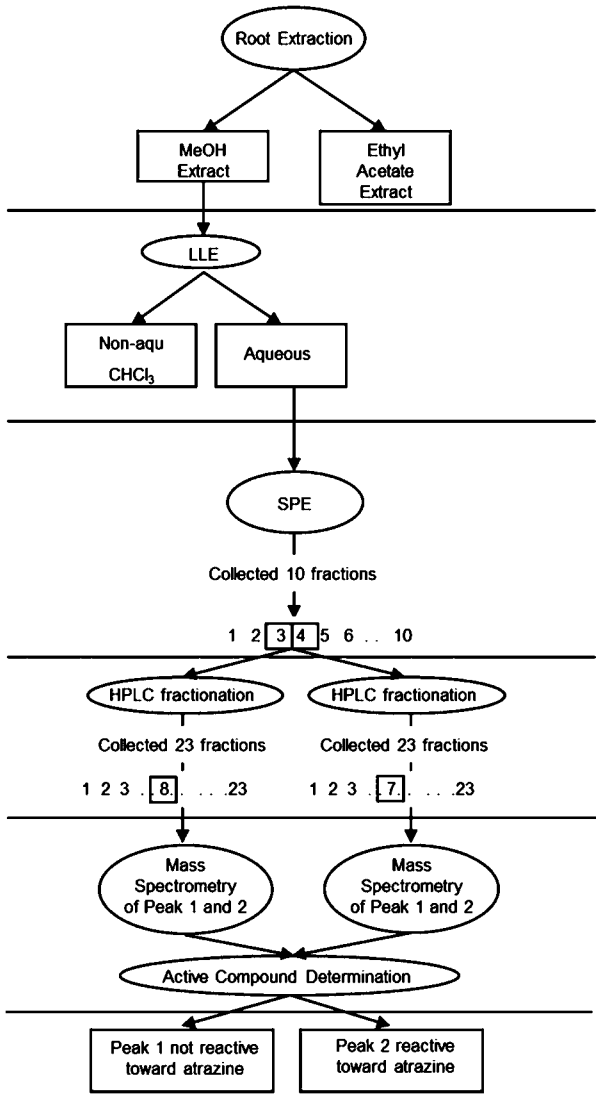

Figure 1. Schematic detailing the extraction, separation, and identification of atrazine-degrading phytochemicals from Eastern gamagrass (*Tripsacum dactyloides*). Corresponding materials and methods paragraph headings appear in the oval shapes. Horizontal lines between sections represent application of an atrazine assay and analysis of fractions for atrazine degradation.

Atrazine Assay and HPLC Analysis. Samples resulting from each level of separation and fractionation as outlined in Figure 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

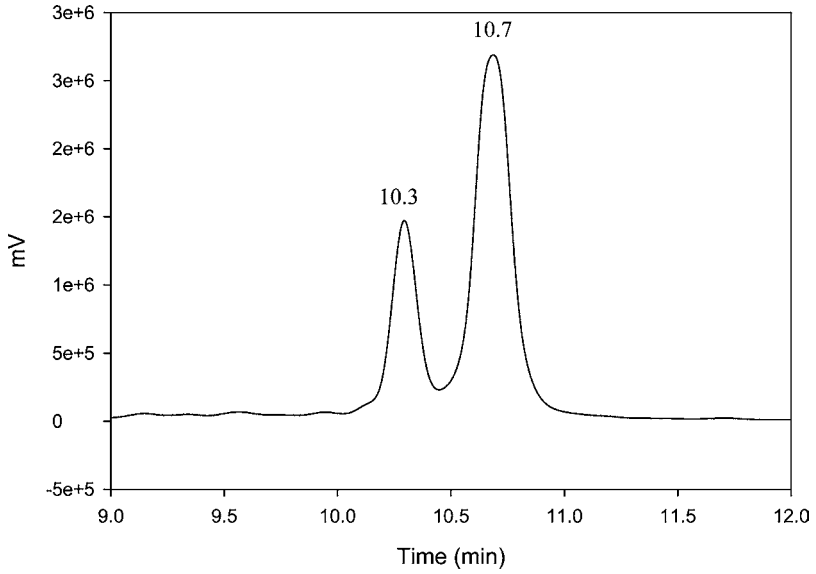

Figure 2. Chromatogram (UV λ = 254 nm) demonstrating peaks 1 and 2 in atrazine-degrading fractions derived from HPLC fractionation of SPE fractions 3 and 4. Retention times: peak 1, 10.3 min; peak 2, 10.7 min.

depending upon the stage of fractionation (Figure 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.[25,26] 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 h. 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 pipets and evaporated to dryness. Samples were resuspended in 1.2 mL of water and analyzed using reverse-phase high-performance liquid chromatography (HPLC) as described by Lin et al.[11] In brief, a Shimadzu LC-10AT VP (Columbia, MD, USA) HPLC equipped with a UV detector was used for analysis. The column was a Columbus C8 (250 × 4.60 mm, 5 μm; Phenomenex, Torrance, CA, USA), 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.

LLE Fractionation. Samples for LLE fractionation were prepared in a similar manner as described for the root extracts. Root samples (100 g wet weight) were blended with 200 mL of 9:1 CH_3OH/H_2O, and 100 mL of CH_3OH was used for the second extraction. To remove the CH_3OH, the filtrates were evaporated to a final volume of 15−20 mL. Concentrated aqueous samples were sonicated for 5−10 min and quantitatively transferred to a separatory funnel with 100 mL (50 mL × 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 LLE was a more appropriate procedure for stopping the reaction. For assays in which 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.,[11] 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 (SPE) Fractionation. SPE cartridges (Applied Separations, Allentown, PA, USA) 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, USA), 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 10 fractions exhibited reasonable activity against atrazine (i.e., >40% degraded). On the basis of this information, only the first 10 SPE fractions were collected. The collection procedure was replicated 10 times. Volumes of the SPE fractions were 6−7 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 was added at the end of the reaction period, and samples were held in cold storage until analysis by HPLC-UV as described for the atrazine assay and HPLC analysis.

HPLC Fractionation. The SPE fractions that exhibited the ability to degrade at least 50% of the atrazine during the atrazine assay (third through eighth SPE fractions; Figure 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.[11]) 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 third through eighth SPE fractions injected. Duplicates were collected from each SPE fraction. The 2 mL samples were evaporated to dryness and resuspended in 150 μL of 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 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 min window from 9.8 to 11.8 min that contained two distinct peaks (peaks 1 and 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) were later shown to contain the same compounds; thus, they were combined for subsequent processing steps.

Mass Spectrometry. Tentative identification of peaks 1 and 2 (Figure 2) was obtained by high-performance liquid chromatography−tandem mass spectrometry with simultaneous diode array detection (HPLC-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, USA). 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.[11] 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 250 °C, the vaporizer temperature was 400 °C, 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,[26] the peaks were tentatively identified. The tentative identifications were further verified against authentic standards. 2-β-D-Glucopyranosyloxy-1,4-benzoxazin-3-one (HBOA-Glc) was purified using methods from Yin et al.,[27] and 2-β-D-glucopyranosyloxy-4-hydroxy-1,4-benzoxazin-3-one (DIBOA-Glc) was purified using methods from Kluge et al.,[28] as cited in Rice et al.,[29] and were provided by Dr. Clifford Rice of USDA-ARS, Beltsville, MD, USA. 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 HPLC-MS/MS-DAD at 255 nm. A molar absorptivity coefficient of 8500 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.[30] Four CH_3OH root extracts were prepared as described under Root Extracts, except that the root and solvent quantities were doubled, and samples were concentrated to ∼15−20 mL. Each sample was analyzed for absorption at 255 nm with HPLC-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 1). The peaks were designated peaks 1 and 2 on the basis of their retention times: 10.3 min for peak 1 and 10.7 min for peak 2 (Figure 2). To separate the peaks into distinct fractions, the sample was separated by HPLC. Fraction collection was performed on the basis of peak slope, and HPLC conditions were as described under HPLC Fractionation except that the H_3PO_4 buffer was omitted from the water/CH_3CN mobile phase. Peak 1 and 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 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 h reaction time, LLE was used to isolate atrazine from the sample matrix. Two milliliters of dichloromethane was added to stop the reaction, and 820 μL of water was 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 resuspended 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 (t_R) 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, whereas 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) was (were) either present in high concentrations or extremely potent in their degradation activity against atrazine.

Although mean atrazine degradation between the two solvents was not significantly different on the basis of 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 LLE 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 redissolved in water to perform the assays without solvent interference, and many or most of the compounds in this fraction likely had limited water solubility. Because 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 phytochem-

icals 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 third and fourth SPE fractions degraded 95.8 ± 5.6 and 99.8 ± 0.32% of the atrazine, respectively (Figure 3). Atrazine degradation in the fifth through eighth SPE fractions ranged from 52.1 to 90.6% of the added atrazine, whereas the 1st, 2nd, 9th, and 10th fractions degraded <36%. Because the third through eighth fractions degraded >50% of the atrazine, they were selected for further fractionation and testing.

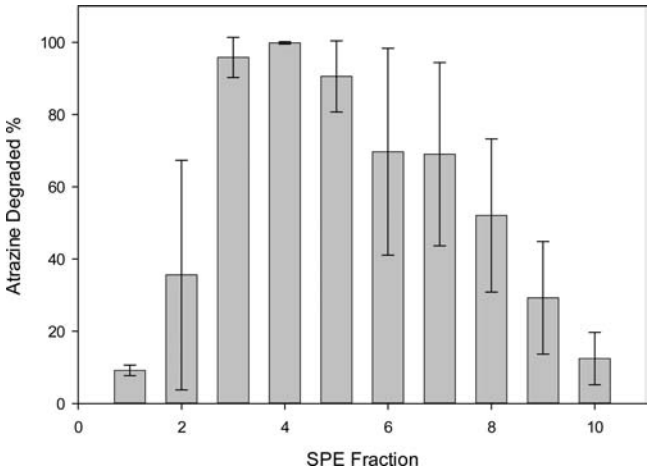

Figure 3. Atrazine degradation exhibited by the 10 fractions collected using SPE. Error bars represent standard deviation. n = 10 for SPE fractions 3−8, and n = 9 for SPE fractions 1, 2, 9, and 10.

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 4). Of the added atrazine, SPE fraction 3, HPLC fraction 8, degraded 68 ± 4.2% (range) and SPE fraction 4, HPLC 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 nonreactive relative to atrazine. The HPLC fractions derived from SPE fractions 5−8 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), and these compounds were initially designated peaks 1 and 2 prior to identification.

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

The HPLC-UV chromatograms of peak 2, with a t_R of 12.65 min, closely matched that of the main peak (t_R = 12.63 min) in the Bx hydroxamic acid standard of DIBOA-Glc (Figure 5a,b). 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 (Figure 5c,d) and that previously reported for DIBOA-Glc in the literature.[26] Product ions from the precursor ion spectra of root extract and the standard yielded m/z 164 and 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 (Figure 5e,f). Loss of the glucose moiety (180 mu) less a water 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 303, respectively. On the basis of the data in Figure 5a−f, the compound in peak 2 was identified as DIBOA-Glc.

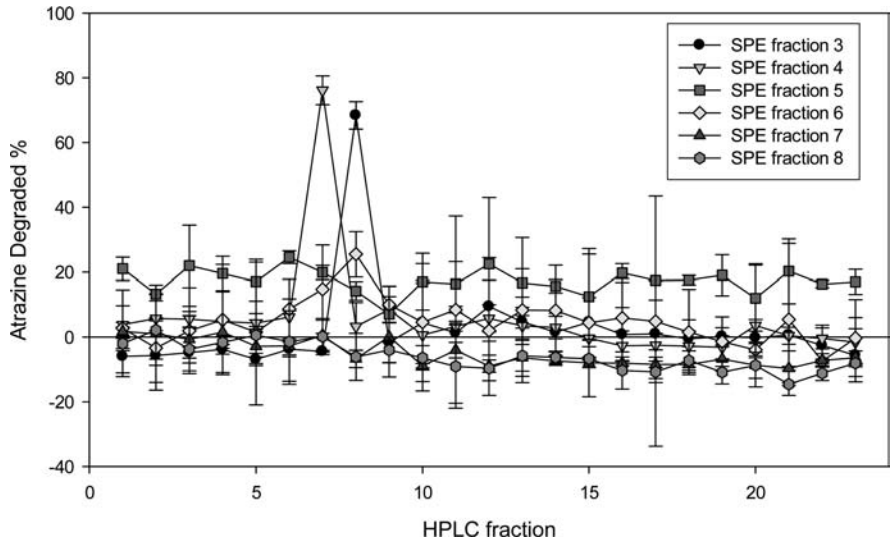

Figure 4. Atrazine degradation exhibited by fractions produced from HPLC separation of SPE master fractions 3−8. Error bars represent the range around the average. n = 2.

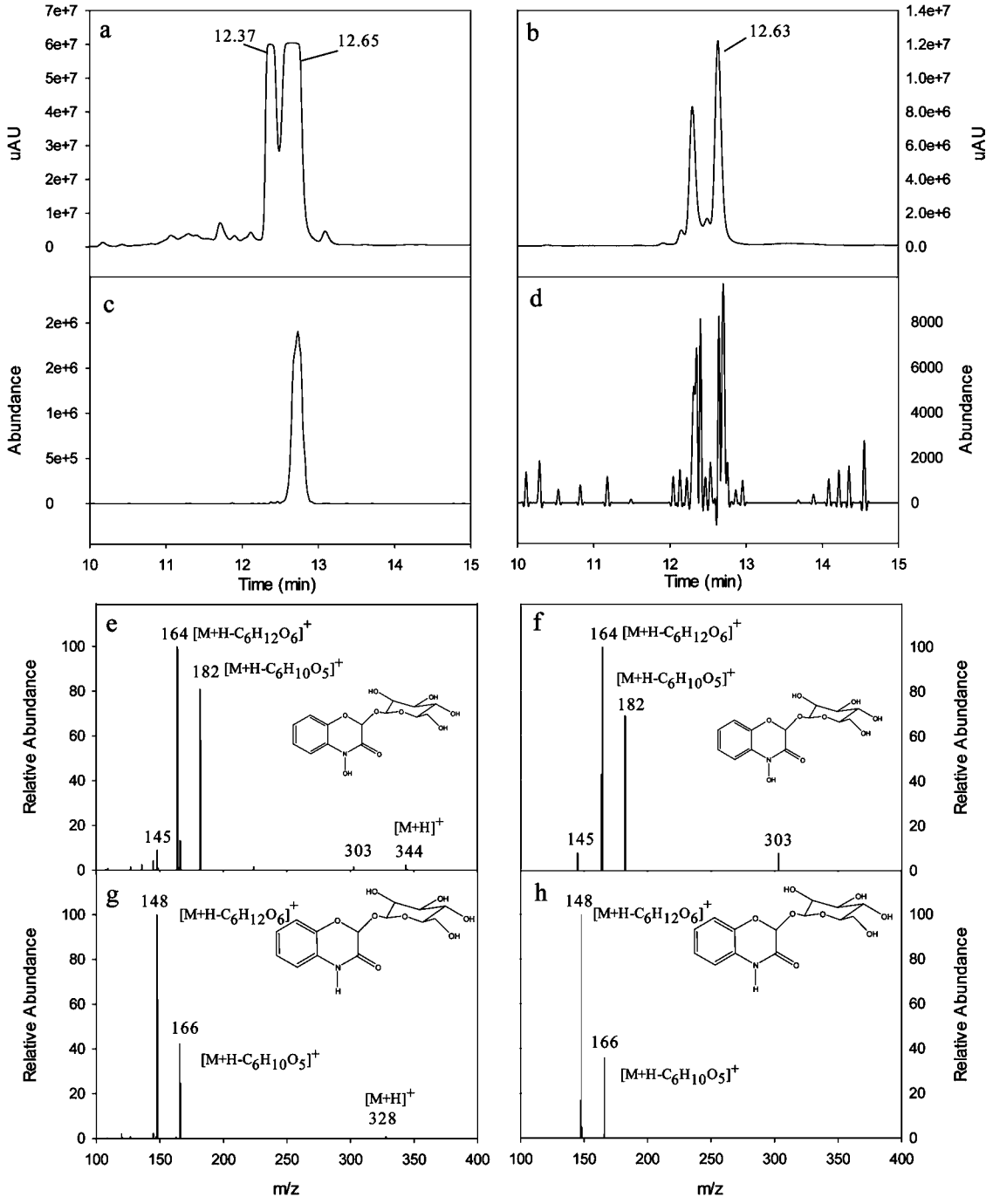

Figure 5. Tenative identification of peaks 1 and 2 from root extract samples from (a) UV chromatogram of root extracted sample (peak 1 t_R = 12.37, peak 2 t_R = 12.65), (b) UV chromatogram from authentic DIBOA-Glc standard (t_R = 12.63), (c) XIC for m/z 344 from root extracted sample, (d) XIC for m/z 344 from authentic DIBOA-Glc standard, (e) MS/MS spectrum $[M + H]^+$ = 344 from root extracted sample, (f) MS/MS spectrum $[M + H]^+$ = 344 from authentic DIBOA-Glc standard, (g) MS/MS spectrum $[M + H]^+$ = 328 from root extract sample, and (h) MS/MS spectrum $[M + H]^+$ = 328 from HBOA-Glc authentic standard.

In the root extracts and the authentic material, the HPLC-UV chromatogram of peak 1 showed a t_R of 12.37 min (Figure 5a), and the HPLC-MS XIC for peak 1 (not shown) had a pseudomolecular ion, $[M + H]^+$, of m/z 328 at the same t_R. The pseudomolecular ion mass also corresponded to data in the literature for the benzoxazinoid lactam HBOA-Glc.[26] The fragment ion spectrum for peak 1 was very similar to the spectra of DIBOA-Glc (Figure 5e,g), 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 (Figure 5g,h). 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 t_R,

dx.doi.org/10.1021/jf402271h | J. Agric. Food Chem. 2013, 61, 8026−8033

HPLC-MS XIC, and fragment ion spectra) peak 1 was identified as HBOA-Glc.

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 and 240 ± 74 mg kg^{-1}, respectively) (UV data in Figure 5a represent one-fifth 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 3 times more concentrated than HBOA-Glc, and both compounds were present in millimolar concentrations (DIBOA-Glc, 3.46 ± 0.02 mM; 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.[18,25,31−33] However, in some cases the range of reported concentrations for Bx compounds spans 4 orders of magnitude even from leaves of the same plant.[33] 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 1980s, several studies in the literature have reported the occurrence of Bx compounds in *Tripsacum*.[13−15] 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[15] 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[15] 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.[13] 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 reactivities toward atrazine were markedly different. The presence of the nucleophilic hydroxamic acid moiety in DIBOA-Glc was apparently critical to its reactivity toward atrazine, whereas 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.[11] Although 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.

AUTHOR INFORMATION

Corresponding Author

*(C.D.W.) E-mail: cdw535@mail.missouri.edu. Phone: (573) 882-1114, ext. 317.

Funding

Funding for this research was provided by the University of Missouri Research Board, Center for Agroforestry, and USDA-ARS Cropping Systems and Water Quality Unit. Mention of specific companies, products, or trade names is made only to provide information to the reader and does not constitute endorsement by the USDA-ARS.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank the USDA−Agricultural Research Service Columbia, MO, staff and students that made this project possible. We especially thank Dr. Clifford Rice, USDA-ARS, Beltsville, MD, who kindly provided Bx standards, and Bettina Coggeshall and Joe Absheer, for technical and analytical support.

ABBREVIATIONS USED

Bx, benzoxazinoid; EG, Eastern gamagrass; DIBOA-Glc, 2-β-D-glucopyranosyloxy-4-hydroxy-1,4-benzoxazin-3-one; CH_3CN, acetonitrile; CH_3OH, methanol; H_3PO_4, phosphoric acid; HBOA-Glc, 2-β-D-glucopyranosyloxy-1,4-benzoxazin-3-one; HPLC-UV, high-performance liquid chromatography with ultraviolet detection; HPLC-MS/MS-DAD, high-performance liquid chromatography-tandem mass spectrometry with diode array detection; LLE, liquid/liquid extraction; t_R, retention time; SPE, solid-phase extraction; Tukey's HSD, Tukey's honest significant difference; XIC, extracted ion chromatogram

REFERENCES

(1) Battaglin, W.; Thurman, E. M.; Kalkhoff, S. J.; Porter, S. D. Herbicides and transformation products in surface waters of the Midwestern United States. *J. Am. Water Resour. Assoc.* **2003**, *39* (4), 743−756.

(2) Fallon, J. D.; Tierney, D. P.; Thurman, E. M. Movement of atrazine and deethylatrazine. *J. Am. Water Works Assoc.* **2002**, *94* (11), 54−54.

(3) Lerch, R. N.; Donald, W. W.; Li, Y.-X.; Alberts, E. E. Hydroxylated atrazine degradation products in a small Missouri stream. *Environ. Sci. Technol.* **1995**, *29* (11), 2759−2768.

(4) Lerch, R. N.; Sadler, E. J.; Sudduth, K. A.; Baffaut, C.; Kitchen, N. R. Herbicide transport in Goodwater Creek Experimental Watershed: I. long-term research on atrazine. *J. Am. Water Resour. Assoc.* **2011**, *47* (2), 209−223.

(5) Squillace, P. J.; Thurman, E. M. Herbicide transport in rivers: importance of hydrology and geochemistry in non-point source contamination. *Environ. Sci. Technol.* **1992**, *26* (3), 538−545.

(6) Thurman, E. M.; Goolsby, D. A.; Meyer, M. T.; Mills, M. S.; Pomes, M. L.; Koplin, D. W. A reconnaissance study of herbicides and

their metabolites in surface water of the Midwestern United States using immunoassay and gas chromatography/mass spectrometry. *Environ. Sci. Technol.* **1992**, *26*, 2440−2447.
(7) Gaynor, J. D.; MacTavish, D. C.; Findlay, W. I. Atrazine and metolachlor loss in surface and subsurface runoff from three tillage treatments in corn. *J. Environ. Qual.* **1995**, *24* (2), 246−256.
(8) Ghidey, F.; Baffaut, C.; Lerch, R. N.; Kitchen, N. R.; Sadler, E. J.; Sudduth, K. A. Herbicide transport to surface runoff from a claypan soil: scaling from plots to fields. *J. Soil Water Conserv.* **2010**, *65* (3), 168−179.
(9) Lin, C. H.; Lerch, R. N.; Garrett, H. E.; George, M. F. Incorporating forage grasses in riparian buffers for bioremediation of atrazine, isoxaflutole and nitrate in Missouri. *Agrofor. Syst.* **2004**, *63* (1), 91−99.
(10) Lin, C. H.; Lerch, R. N.; Garrett, H. E.; George, M. F. Bioremediation of atrazine-contaminated soil by forage grasses: transformation, uptake, and detoxification. *J. Environ. Qual.* **2008**, *37*, 196−206.
(11) Lin, C. H.; Lerch, R. N.; Kremer, R. J.; Garrett, H. E. Stimulated rhizodegradation of atrazine by selected plant species. *J. Environ. Qual.* **2011**, *40*, 1113−1121.
(12) Brands, E.; Rajagopal, R. Economics of place-based monitoring under the safe drinking water act, part I: spatial and temporal patterns of contaminants, and design of screening strategies [electronic resource]. *Environ. Monit. Assess.* **2008**, *143* (1−3), 75−89.
(13) Frey, M.; Schullehner, K.; Dick, R.; Fiesselmann, A.; Gierl, A. Benzoxazinoid biosynthesis, a model for evolution of secondary metabolic pathways in plants. *Phytochemistry* **2009**, *70* (15−16), 1645−1651.
(14) Marcacci, S.; Raveton, M.; Ravanel, P.; Schwitzguébel, J.-P. Conjugation of atrazine in vetiver (*Chrysopogon zizanioides* Nash) grown in hydroponics. *Environ. Exp. Bot.* **2006**, *56* (2), 205−215.
(15) Niemeyer, H. M. Hydroxamic acids (4-hydroxy-1,4-benzoxazin-3-ones), defence chemicals in the Gramineae. *Phytochemistry* **1988**, *27*, 3349−3358.
(16) Rice, C. P.; Cai, G.; Teasdale, J. R. Concentrations and allelopathic effects of benzoxazinoid compounds in soil treated with rye (*Secale cereale*) cover crop. *J. Agric. Food Chem.* **2012**, *60* (18), 4471−4479.
(17) Fomsgaard, I. S.; Mortensen, A. G.; Carlsen, S. C. K. Microbial transformation products of benzoxazolinone and benzoxazinone allelochemicals: a review. *Chemosphere* **2004**, *54* (8), 1025−1038.
(18) Gianoli, E.; Niemeyer, H. DIBOA in wild Poaceae: sources of resistance to the Russian wheat aphid (*Diuraphis noxia*) and the greenbug (*Schizaphis graminum*). *Euphytica* **1998**, *102* (3), 317−321.
(19) Niemeyer, H. M. Hydroxamic acids derived from 2-hydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one: key defense chemicals of cereals. *J. Agric. Food Chem.* **2009**, *57* (5), 1677−1696.
(20) Niemeyer, H. M.; Copaja, S. V.; Barria, B. N. The Triticeae as sources of hydroxamic acids, secondary metabolites in wheat conferring resistance against aphids. *Hereditas* **1992**, *116*, 295−299.
(21) Macias, F.; Marin, D.; Oliveros-Bastidas, A.; Castellano, D.; Simonet, A. M.; Molinillo, J. M. G. Structure-activity realtionships (SAR) studies of benzoxazinones, their degradation products and analogues, phytotoxicity on standard traget species (STS). *J. Agric. Food Chem.* **2005**, *53*, 535−548.
(22) Castelfranco, P.; Brown, M. S. Purification and properties of the simazine-resistance factor of *Zea mays*. *Weeds* **1962**, *10* (2), 131−136.
(23) Castelfranco, P.; Chester, L. F.; Deutsch, D. B. Non-enzymatic detoxification of 2-chloro-4,6-bis(ethylamino)-s-triazine (simazine) by extracts of *Zea mays*. *Weeds* **1961**, *9* (4), 580−591.
(24) Yang, H. *Enhanced Rhizodegradation of Munitions Explosives and Degradates by Selected Native Grass Species*. M.S. Thesis, Univsersity of Missouri, Columbia, 2010.
(25) Baumeler, A.; Hesse, M.; Werner, C. Benzoxazinoids-cyclic hydroxamic acids, lactams and their corresponding glucosides in the genus *Aphelandra* (Acanthaceae). *Phytochemistry* **2000**, *53* (2), 213−222.
(26) Cambier, V.; Hance, T.; de Hoffmann, E. Non-injured maize contains several 1,4-benzoxazin-3-one related compounds but only as glucoconjugates. *Phytochem. Anal* **1999**, *10* (3), 119−126.
(27) Yin, H.; Zhang, S.; Luo, X.; Liu, Y. Preparative isolation and purification of two benzoxazinoid glucosides from *Acanthus ilicifolius* L. by high-speed counter-current chromatography. *J. Chromatogr., A* **2008**, *1205* (1−2), 177−181.
(28) Kluge, M.; Schneider, B.; Sicker, D. Diastereoselective synthesis of the benzoxazinone acetal glucoside ent-GDIMBOA: the first enantiomer of a natural acetal glucoside. *Carbohydr. Res.* **1997**, *298* (3), 147−152.
(29) Rice, C.; Park, Y.; Adam, F.; Abdul-Baki, A.; Teasdale, J. Hydroxamic acid content and toxicity of rye at selected growth stages. *J. Chem. Ecol.* **2005**, *31* (8), 1887−1905.
(30) Bailey, B. A.; Larson, R. L. Hydroxamic acid glucosyltransferases from maize seedlings. *Plant Physiol.* **1989**, *90* (3), 1071−1076.
(31) Hofman, J.; Hofmanova, O. 1,4-Benzoxazine derivatives in plants. *Eur. J. Biochem.* **1969**, *8*, 109−112.
(32) Larsen, E.; Christensen, L. P. Simple method for large scale isolation of the cyclic arylhydroxamic acid DIMBOA from maize (*Zea mays* L.). *J. Agric. Food Chem.* **2000**, *48* (6), 2556−2558.
(33) Lyons, P. C.; Hipskind, J. D.; Wood, K. V.; Nicholson, R. L. Separation and quantification of cyclic hydroxamic acids and related compounds by high-pressure liquid chromatography. *J. Agric. Food Chem.* **1988**, *36* (1), 57−60.