


Application of Direct Analysis in Real Time–High Resolution Mass Spectrometry to Investigations of Induced Plant Chemical Defense Mechanisms—Revelation of Negative Feedback Inhibition of an Alliinase

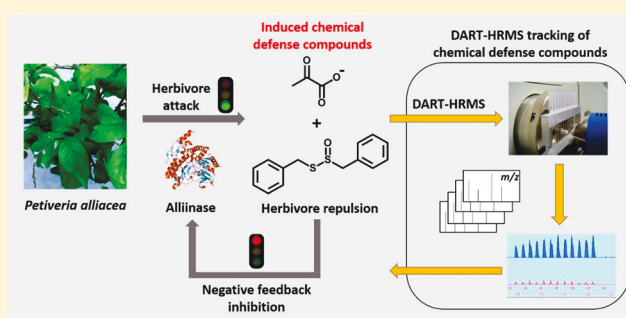
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Supporting Information

ABSTRACT: Several plants of agricultural and medicinal importance utilize defense chemistry that involves deployment of highly labile, reactive, and lachrymatory organosulfur molecules. However, this chemistry is difficult to investigate because the compounds are often short-lived and prone to degradation under the conditions required for analysis by common analytical techniques. This issue has complicated efforts to study the defense chemistry of plants that exploit the use of sulfur in their defense arsenals. This work illustrates how direct analysis in real time–high resolution mass spectrometry (DART-HRMS) can be used to track organosulfur defense compound chemistry under mild conditions.

Petiveria alliacea was used as a model plant that exploits the enzyme alliinase to generate induced organosulfur compounds in response to herbivory. Tracking of the organosulfur compounds it produces and quantifying them by DART-HRMS using isotopically labeled analogues revealed a feedback inhibition loop through which the activities of the alliinase are stymied shortly after their activation. The results show that the downstream thiosulfinate products petivericin (100 μ M) and pyruvate (8.4 mM) inhibit alliinase activity by 60% and 29%, respectively, after 1 h, and a mixture of the two inhibited alliinase activity by 65%. By 2 h, alliinase activity in the presence of these alliinase-derived products had ceased completely. Because thiosulfinate, pyruvate, and lachrymatory sulfine compounds are produced via the same alliinase-derived sulfenic acid intermediate, the inhibition of alliinase activity by increasing concentrations of downstream products shows how production of these defense compounds is modulated in real time in response to a tissue breach. These findings provide a framework within which heretofore unexplained phenomena observed in the defense chemistry of *P. alliacea*, onion, garlic, and other plants can be explained, as well as an approach by which to track labile compounds and enzymatic activity by DART-HRMS.



Plants have evolved a wide range of mechanisms to ward off herbivores and microbial infections. Those that are chemically based can be either constitutive (i.e., the defense molecules are always present) or induced, meaning that they are generated and deployed in response to tissue injury. Plant chemical defense mechanisms that exploit induced formation of organosulfur compounds are difficult to study by many conventional methods because of the lability of the organosulfur compounds themselves. For example, GC/MS methods that are commonly used to track the formation of defense compounds result in artifacts derived from reactions that organosulfur compounds undergo in the GC injection port.^{1–10} This leads to incorrect conclusions not only about the identities of the molecules present but also about the details of the steps in the chemical defense strategy. Method-induced artifact formation continues to hamper studies of the mechanisms by which a number of well-known plants of culinary and medicinal importance initiate and curtail the

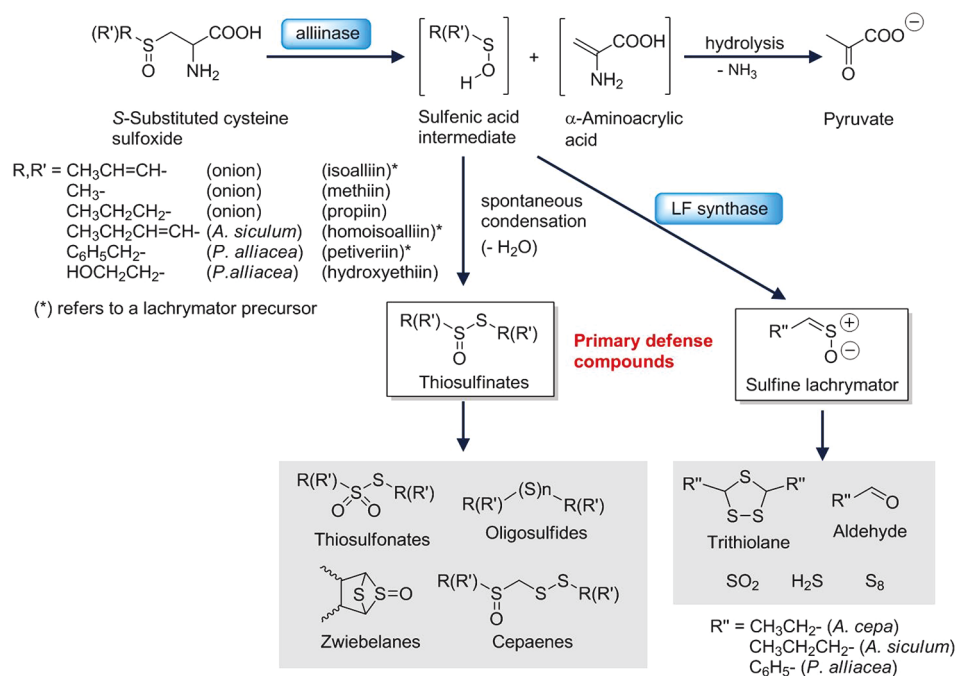
formation of these molecules when tissue injury occurs. Plants that utilize “*Allium* chemistry” serve as a case in point. Onion (*Allium cepa*), garlic (*A. sicutum*), and the Amazonian medicinal plant *Petiveria alliacea* are species that utilize thiosulfates ($R-S(=O)S-R$) and lachrymatory sulfines ($R-CH=S=O$) as defense compounds.^{11–15} As is illustrated in Scheme 1, they are formed as part of a complex network of *S*-substituted cysteine sulfoxide-derived defensive secondary metabolites that include vesicant thiosulfates, oligosulfides, thiosulfonates, trithiolanes, cepaenes, zwibelanes, aldehydes, elemental sulfur, and other small inorganic signaling molecules such as H_2S and SO_2 .^{16,17} These compounds are produced when the previously compartmentalized pyridoxal phosphate (PLP)-dependent C–S lyase (i.e., alliinase) is exposed to

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Scheme 1. General Outline of Defense Compound Production Chemistry Illustrated for Onion (*Allium cepa*), *Allium sicutum*, and *Petiveria alliacea*^a



^aExposure of cytoplasmic S-substituted cysteine sulfoxide derivatives to vacuolar alliinase results in cleavage to form sulfenic acids and α -aminoacrylic acid. The α -aminoacrylic acid is hydrolyzed to pyruvate, whereas the sulfenic acid can either (1) condense with a second molecule of sulfenic acid (with loss of water) to form symmetrical or unsymmetrical thiosulfonates or (2) undergo transformation by lachrymatory factor synthase (LFS) to a sulfine lachrymator. Both the lachrymators and the thiosulfonates undergo further transformations to yield the derivatives shown.

constitutively present S-substituted cysteine sulfoxides as a consequence of tissue disruption. As illustrated in Scheme 1, lachrymator production in response to herbivore challenge begins with exposure of cytoplasmic cysteine sulfoxide precursors to previously compartmentalized vacuolar alliinase enzymes. The first stable and easily isolatable products formed from this interaction are pyruvate and thiosulfinate compounds. The pyruvate is formed from α -aminoacrylic acid, whereas the thiosulfonates occur through condensation of two molecules of the sulfenic acid intermediate that is initially formed by alliinase-mediated cleavage of S-substituted cysteine sulfoxides. The presence of lachrymatory factor synthase (LFS)^{15,18–20} is crucial for the production of the volatile sulfine lachrymators. The lachrymators are unstable in the aqueous medium in which they are formed and are quickly hydrolyzed to the corresponding aldehydes.²¹

The organosulfur derivatives formed through the action of alliinase are reactive and susceptible to attack by oxygen, nitrogen, and sulfur nucleophiles.^{22–26} Extracts of plants that produce these compounds are known to be destructive to living tissue and to cause contact dermatitis, blisters, and chemical burns.^{27–32} A number of studies have also shown that some of these compounds elicit painful inflammatory responses in animal predators through activation of TRPA1 and TRPV1, two temperature-activated ion channels that are present in the pain-sensitive neurons that activate the mouth.^{33–37} For these reasons, the utilization of this type of weaponry in plants is potentially problematic because of the collateral damage it can cause. The electrophilicity of the various defense compounds makes them corrosive and/or highly reactive. This, coupled with their nonspecificity toward

reactions with cellular nucleophiles, renders plant cells at the site of injury susceptible to indiscriminate attack by the very defense compounds produced to ward off predators. This scenario presents plants that utilize alliinase-mediated defense chemistry with a dilemma: how to mount an effective assault against a diverse population of attackers (e.g., vertebrate herbivores or insects), while minimizing self-damage caused by the continued formation of corrosive lachrymators and other electrophilic organosulfur compounds. The threat of incursions by pathogenic microbes at the site of injury adds another layer of complexity to the problem. Historically, the monitoring of how formation of labile organosulfur defense compounds is curtailed has been challenged because of analysis method-induced transformations that form artifacts.

Direct analysis in real time—high resolution mass spectrometry (DART-HRMS) and proton transfer reaction mass spectrometry (PTR-MS) have been shown to be well-suited for the detection of labile organosulfur compounds, without promoting their degradation or the formation of artifacts that has been observed by GC/MS.^{13,38–41} For example, PTR-MS has been used to detect and track volatile organosulfur compounds emitted from several Brassica species in response to herbivory.^{38,39,41,42} In addition, Samudrala et al. have reported optimized conditions for the observation of such compounds in plants using this technique.⁴⁰ DART-HRMS was used to demonstrate for the first time the formation of fleeting sulfenic acid intermediates formed in onion and garlic on tissue injury.¹³ It has also been used to reveal how the roots of *Mimosa pudica* emit a spray of noxious organosulfur compounds in response to touch.⁴³ We report here how DART-HRMS and isotopically labeled precursor and product

molecules can be used to readily track and quantify the formation of induced labile organosulfur defense compounds in a manner that reveals information about the impact of defense molecules on the proteins that mediate their formation. Following this approach and using *P. alliacea* as a model, DART-HRMS monitoring of plant-derived alliinase-mediated breakdown of a precursor cysteine sulfoxide illustrated a novel negative feedback inhibition by which molecules downstream of alliinase ultimately inhibit its function.

EXPERIMENTAL SECTION

Materials. Pyruvate- d_3 was purchased from Cambridge Isotopes Laboratories, Inc. (Tewksbury, MA). *m*-Chloroperoxybenzoic acid (*m*-CPBA) was purchased from Fisher Scientific (Hampton, NH). Coomassie (Bradford) Protein Assay kit was purchased from Thermo Scientific (Rockford, IL). Biotage SNAP Ultra Cartridges were purchased from Biotage (Charlotte, NC). Methylene chloride and hexanes were acquired from Pharmco-Aaper (Brookfield, CT). Dibenzyl disulfide- d_{14} was synthesized from benzyl chloride- d_7 . Its synthetic route is outlined in [Supporting Information Figure S-1](#), and its characterization is also described in the [Supporting Information](#). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO). *P. alliacea* alliinase/LFS were extracted according to the method of Musah et al.,²⁰ with minor modifications. Whole fresh *P. alliacea* plants were obtained from Native Habitat Landscaping (Vero Beach, FL).

Confirmation of Alliinase Activity in *P. alliacea* Roots. Woody *P. alliacea* roots were harvested from the plant and, after tissue disruption, were immediately analyzed using DART-HRMS in both positive and negative ion modes at 350 °C. Spectra were acquired by suspending the samples in the open-air space between the ion source and the mass spectrometer inlet.

Mass Spectral Acquisition and Data Processing. DART-HRMS mass spectra of the synthesized compounds and calibration standards were acquired using a DART-SVP ion source (IonSense, Saugus, MA) coupled to a JEOL AccuTOF high resolution time-of-flight mass spectrometer (JEOL USA, Peabody, MA). Petivericin and petiveriin calibration standards were analyzed in positive-ion mode, while the pyruvate calibration standard was analyzed in negative ion mode. The parameters for the DART ion source were as follows: grid voltage, 250 V; and gas heater temperature, 350 °C. The settings for the mass spectrometer were as follows: ring lens voltage, 5 V (−5 V for negative ion mode); orifice 1 voltage, 20 V (−20 V for negative ion mode); orifice 2 voltage, 5 V (−5 V for negative ion mode); and peak voltage, 600 V. Spectra were collected over the m/z range 60–800 at a rate of 1 spectrum/s. The ion source was operated with ultra high-purity helium (Airgas, Albany, NY) at a flow rate of 2 L/min, and the resolving power of the mass spectrometer was 6000 full width at half maximum (fwhm). A 12 DIP-it sampler (IonSense, Saugus, MA) was used to automate the analysis of the calibration standards (described below and in [Figure S-2](#)). Poly(ethylene glycol) (PEG; average molecular weight 600) was analyzed with every acquired spectrum as a standard for accurate mass determinations. TSSPro 3 software (Shrader Analytical, Detroit, MI) was used for data processing including averaging, centroiding, background subtraction, and integration.

Semiautomated Analysis Protocol. A 12 DIP-it sampler (IonSense, Saugus, MA) was used to automate the analysis of the calibration standards (described below and in [Figure S-2](#)). Utilization of a linear rail system within which the DIP-it tips were mounted allowed for reproducibility between sample replicates. To detect the analytes of interest by DART-HRMS, it was determined that amounts of ≥ 0.786 ng were required, because samples of less than this amount did not yield ion chromatograms that were consistent enough to enable quantification. In this case, this was generally equivalent to 1.5 μ L of a 2 μ M solution of the calibration standards or the protein extract. This volume was deposited onto the tip of the capillary tube of each DIP-it tip. The tips were mounted at the same height in the linear rail with the tips oriented so that the applied sample was facing the DART gas stream. Once the solution was applied to the tips, it was allowed to dry before DART-HRMS analysis, in order to avoid the issue of the liquid wrapping around the tips when the tips entered the gas stream. This enabled the acquisition of highly consistent peak area ratio results. A constant linear rail speed of 1 mm/s eliminated sample carryover between replicates, in addition to providing baseline separation of signals in the total ion chromatograms. The linear rail was positioned in the middle of the 4 cm space between the mass spectrometer inlet and the ion source. Adjustment of this distance between experimental runs was found to introduce inconsistencies in peak intensities, which precluded accurate quantification of analytes.

Preparation of Standard Curves Using Calibration Standards. A petivericin stock solution of 100 ppm was prepared in buffer A (20 mM phosphate buffer at pH 8.0 containing 25 μ M PLP and 6 mM petiveriin) with 20 ppm internal standard petivericin- d_{14} . Serial dilutions of the 100 ppm stock solution were used to prepare 10 sets of calibration standard samples ranging from 10 to 100 ppm. A pyruvate stock solution of 500 ppm was prepared in buffer A with 100 ppm internal standard pyruvate- d_3 . Serial dilutions of the 500 ppm stock solution were used to prepare 11 sets of calibration standard samples ranging from 25 to 400 ppm. A 500 ppm petiveriin stock solution was prepared in buffer A with 200 ppm of the internal standard *S*-methyl-L-cysteine. Serial dilutions of the 500 ppm stock solution were used to prepare 11 sets of calibration standard samples ranging from 1 to 500 ppm. The total volume of each calibration standard sample was 500 μ L, and it was analyzed in triplicate. Peak integration was performed using the peaks for petivericin (m/z 263.0564), petivericin- d_{14} (m/z 277.1443), pyruvate (m/z 87.0082), pyruvate- d_3 (m/z 90.0270), petiveriin (m/z 228.0694), and *S*-methyl-L-cysteine (m/z 136.0432). The DART-HRMS peak area ratios (PAR) of petivericin to petivericin- d_{14} , pyruvate to pyruvate- d_3 , and petiveriin to *S*-methyl-L-cysteine were used in the generation of calibration curves to correct for variation in the instrument response to the samples. The PAR for petivericin, pyruvate, and petiveriin are illustrated in [Tables S1, S2, and S3](#), respectively, and the standard curves for petivericin, pyruvate, and petiveriin are presented in [Figure S-3](#).

Alliinase Extraction and Determination of Protein Concentration. Alliinase was extracted using a previously reported protocol²⁰ with the following modifications: (i) the extraction buffer did not contain 2-mercaptoethanol (BME) and polyvinylpyrrolidone (PVPP); and (ii) the protein extract after dialysis was concentrated using Millipore centrifugal filter units (Burlington, MA) with a molecular weight cut off of 10

kDa. Assessment of the concentration of the protein acquired from *P. alliaceae* roots was performed using the Coomassie (Bradford) Protein Assay kit according to manufacturer specifications. The protein extract concentration was determined to be $\sim 4 \mu\text{g/g}$ of fresh roots.

Kinetics Studies of Alliinase Activity. The time frame at room temperature during which the alliinase was active was determined by monitoring the production of pyruvate and the sulfine phenylmethanethial-S-oxide (PMTSO) using DART-HRMS when 6 mM of the petiveriin substrate was exposed to alliinase in 20 mM phosphate buffer at pH 8.0 containing 25 μM PLP. DART-HRMS spectra were acquired at 15, 30, 45, 60, 75, 90, 105, 120, 150, and 180 min. The samples were analyzed in triplicate. The productions of pyruvate and PMTSO were determined by total ion counts. The total ion counts of these compounds and their standard deviations are illustrated in Tables S4 and S5.

Determination of the Factors Affecting *P. alliaceae* Alliinase Activity. Alliinase activity was monitored using petiveriin as the substrate. The 500 μL reaction mixture was composed of 20 mM phosphate buffer at pH 8.0 containing 6 mM petiveriin, 25 μM PLP, and 40 $\mu\text{g/mL}$ alliinase/LFS (buffer B). The solution was incubated for 1 h at room temperature and then analyzed using the protocol described in the previous section. The concentrations of pyruvate and petivericin were determined by using the standard curves described previously. Pyruvate (concentrations ranging from 0.5 to 8.4 mM) was added to 500 μL of buffer B. The influence of pyruvate was determined by monitoring the formation of petivericin over the course of 1 h by DART-HRMS. The petivericin concentration was determined from the petivericin/petivericin- d_{14} standard curve. Petivericin (concentrations ranging from 2 to 100 μM) was added to 500 μL of buffer A. The influence of petivericin was determined by monitoring the formation of pyruvate at 1 h. The pyruvate concentration was determined from the pyruvate/pyruvate- d_3 standard curve. To study the influence of the combination of pyruvate and petivericin on alliinase activity, 4.2 mM pyruvate and 110 μM petivericin were added into 500 μL of buffer B. The influence of a combination of pyruvate and petivericin was determined by monitoring the decrease of the substrate petiveriin over the course of 1 h. Petiveriin consumption was determined using the petiveriin/S-methyl-L-cysteine standard curve. The PAR and relative amounts of petivericin, pyruvate, and petiveriin are illustrated in Tables S6, S7, and S8, respectively.

RESULTS

DART-HRMS-Facilitated Confirmation of Enzymatic Activity of Alliinase/LFS Derived from *P. alliaceae* Roots. The *P. alliaceae* alliinase/LFS complex was isolated from plant roots as described in previous work.²⁰ The protein concentration was determined to be $\sim 4 \mu\text{g/g}$ of fresh roots. We initiated our enzyme-activity studies by confirming the enzyme was active in planta, which was assessed by the observation of substrate (petiveriin) and alliinase-derived products in breached root tissue. Product formation was monitored by DART-HRMS. When freshly cut segments of the plant roots were analyzed by suspending them between the ion source and the mass spectrometer inlet, petiveriin in its protonated form was observed in positive ion mode at m/z 228.0694 (Figure 1A), and the thiosulfinate product petivericin was also observed in its protonated form at m/z 263.0564. Pyruvate $[\text{M}-\text{H}]^-$, another product of this reaction, was

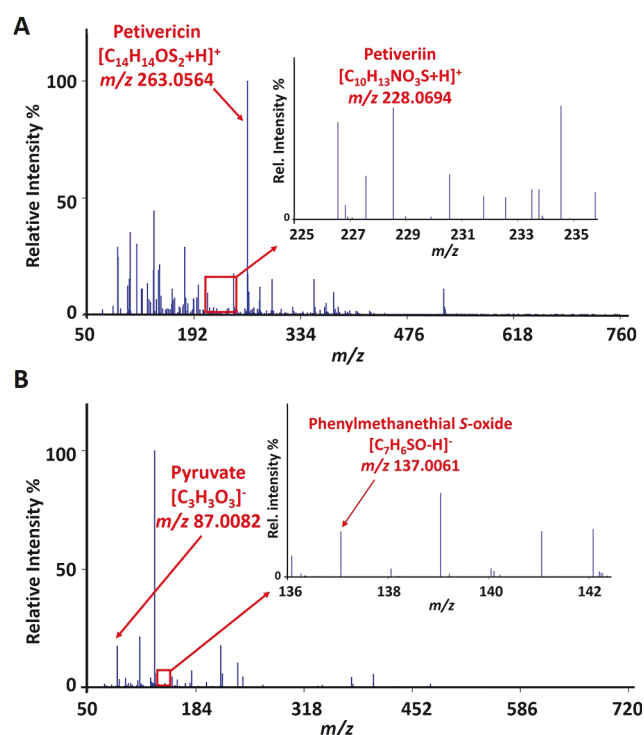


Figure 1. DART-HRMS spectra of injured *Petiveria alliaceae* roots at 350 $^{\circ}\text{C}$. (A) Positive ion mode. Petiveriin and petivericin were detected at m/z 228.0694 and m/z 263.0564, respectively. (B) Negative ion mode. The pyruvate and sulfine products were detected at m/z 87.0082 and m/z 137.0061, respectively.

detected at m/z 87.0082 in negative ion mode (Figure 1B). The lachrymatory sulfine phenylmethanethial-S-oxide (PMTSO) was also observed in negative ion mode at m/z 137.0061. These results confirmed the presence of alliinase/LFS and petiveriin in fresh tissue and that the enzyme complex was functional, because the products petivericin, pyruvate, and PMTSO were all detected.

Determination of Alliinase/LFS Activity at Room Temperature by DART-HRMS Using Isotopically Labeled Analogues. To determine whether (i) alliinase activity decreases over time and (ii) at what time point the enzyme ceases to be active under a given set of conditions, we monitored the formation of pyruvate by DART-HRMS when the enzyme was exposed to petiveriin using the alliinase protein extract obtained from *P. alliaceae* roots. On combining petiveriin with alliinase/LFS at concentrations representative of that observed in fresh roots (6 mM and 40 $\mu\text{g/mL}$, respectively), the ion counts of pyruvate were monitored every 15 min over the first 1.5 h and every 30 min over the next 1.5 h. The results, which are presented in Figure 2, showed a modest increase in pyruvate formation during the first 40 min, a more dramatic increase during the next 40 min, and then a plateau within the second hour (indicative of cessation of enzyme activity). This showed that, over time, alliinase activity was curtailed. PMTSO formation over the same time frame also plateaued, indicating that its production eventually ceased (Figure S-4). To investigate whether it was the buildup of products that impacted alliinase activity, we first investigated the amounts of pyruvate and petivericin that were formed over time when *P. alliaceae* roots were injured. Fresh root-derived alliinase/LFS was exposed to 6 mM petiveriin, and to quantify the products by DART-HRMS, synthesized petivericin- d_{14} and

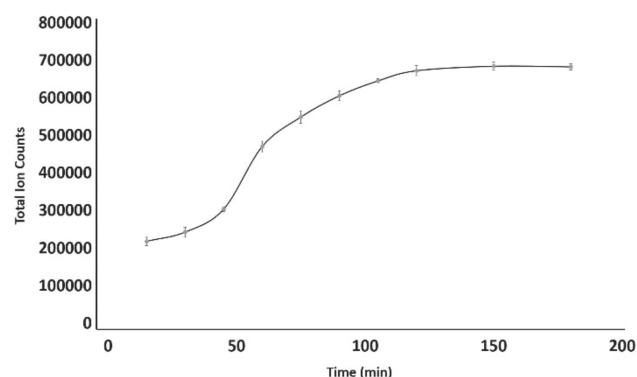


Figure 2. Activity of alliinase at room temperature as a function of time. The reaction was monitored over a period of 180 min. The plot shows a modest increase of pyruvate formation during the first 40 min, a more dramatic increase during the next 40 min, and then a plateau in the second hour (indicative of cessation of alliinase activity).

commercially available pyruvate- d_3 were used as internal standards. The standard curves constructed for the quantification of petivericin and pyruvate were based on plots of the peak area ratios (PARs) of the total ion counts of deuterated and nondeuterated petivericin and pyruvate, versus the known concentrations of the nondeuterated compounds. These plots are presented in Figure S-3 and had R^2 values of 0.9971 and 0.9959, respectively. Using these standard curves, the concentrations of petivericin and pyruvate observed at 1 h were determined to be 110 μM and 4.2 mM, respectively.

Alliinase Activity Is Influenced by a Combination of Small-Molecule Downstream Products. To determine whether products of alliinase-mediated reactions had an effect on alliinase activity, we assessed the influence of increasing concentrations of these compounds on alliinase activity in the protein extract at physiologically relevant concentrations using DART-HRMS. We first monitored the effect of increasing concentrations of pyruvate by monitoring the formation of petivericin when the substrate petiveriin was exposed to alliinase. Compared with the amount of petivericin formed in the absence of added pyruvate, the addition of increasing amounts of pyruvate (from 0.5 to 8.4 mM) resulted in a modest decrease in petivericin formation, from 95% to 71% over 1 h (Figure 3A). On the other hand, increasing concentrations of petivericin (from 2 to 100 μM) resulted in a decrease in the formation of pyruvate from 77% to 40% over 1 h relative to the control where no petivericin was added (Figure 3B). These results showed that pyruvate and petivericin each exerted an inhibitory effect on the alliinase activity. We observed the negative effects of petivericin on the alliinase to be more dramatic than that of pyruvate. The more dramatic effect of this alliinase inhibitor aligns with the finding that a number of onion-derived flavones exert a modest negative impact on onion alliinase activity.⁴⁴

Because both products are formed and are present when alliinase is active in planta, we sought to determine their effect on alliinase activity when present in combination. The impact of these compounds on alliinase was assessed by monitoring the change in the amount of petiveriin starting material (after 1 h) relative to the control when these compounds were exposed to alliinase. The amount of petiveriin observed after 1 h under physiologically relevant conditions served as the control. When 4.2 mM pyruvate and 110 μM petivericin (the amounts that

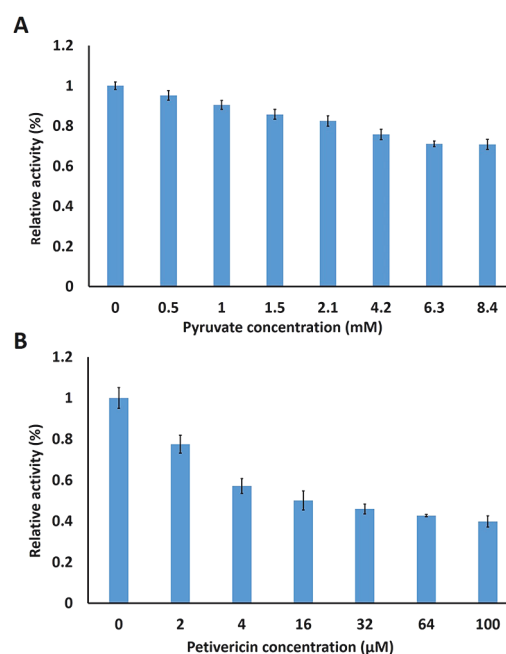


Figure 3. Effects of pyruvate and petivericin on *P. alliacea* alliinase activity. (A) Negative effect of increasing pyruvate concentrations on alliinase activity; (B) negative effect of increasing petivericin concentrations on alliinase activity. The effect of pyruvate on the alliinase was determined by monitoring petivericin production (A). The influence of petivericin on the alliinase was determined by monitoring the formation of pyruvate (B).

were previously determined to be produced after 1 h under physiologically relevant conditions) were exposed to alliinase/LFS separately and as a mixture, the amount of petiveriin starting material that was used up decreased in each case, with the greatest decrease being observed for the mixture. These results are presented in Figure 4. For 4.2 mM pyruvate, the

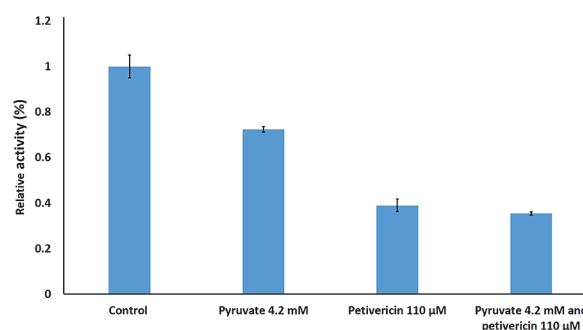
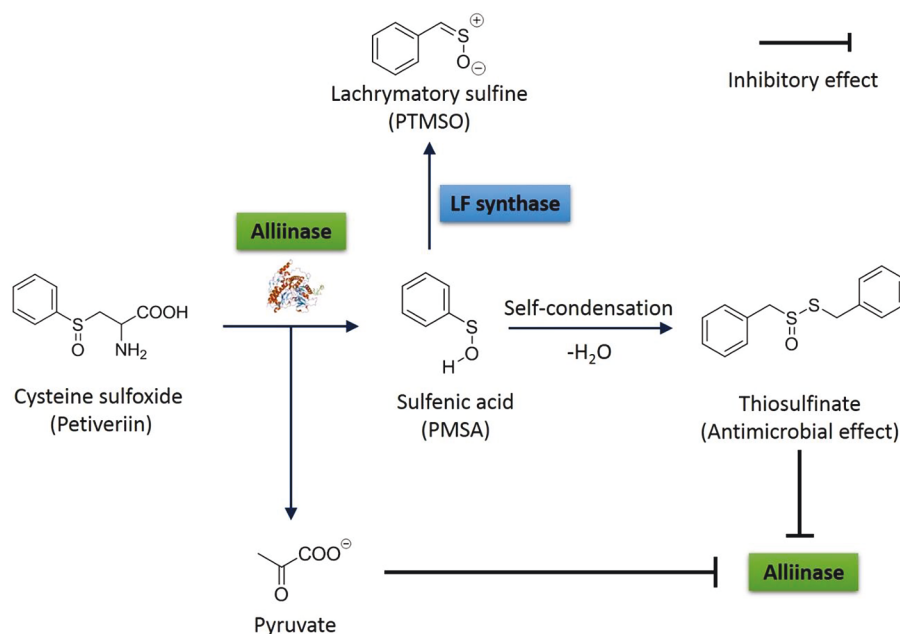


Figure 4. Negative effects of the indicated concentrations of pyruvate and petivericin, as well as a combination of these compounds, on the activity of *P. alliacea* alliinase/LFS. The influences of pyruvate and petivericin were determined by monitoring the consumption of the substrate petiveriin.

activity of alliinase was reduced to 72% that of the control, and when exposed to 110 μM petivericin, the activity was reduced to 39% that of the control. The activity of the alliinase was further reduced to 35% relative to the control when a mixture of 4.2 mM pyruvate and 110 μM petivericin was used. These results indicated that the downstream products pyruvate and petivericin had an inhibitory effect on the activity of alliinase when added individually. Furthermore, when present as a

Scheme 2. Outline of the Feedback Inhibition Loop Involving *P. alliacea* Alliinase^a

^aWithin seconds of wounding, the exposure of petiveriin to an alliinase/LFS complex results in rapid production of the highly irritating lachrymatory sulfine PMTSO, which causes long-lasting and severe irritation to the eyes, nose, and lungs. This serves as a first line of defense in deterring vertebrate herbivores. A second component of the defense strategy involves release of the thiosulfinate petivericin, which serves the dual role of curtailing the growth of microbes at the site of the wound pending healing and inhibiting the alliinase. Increasing amounts of petivericin and pyruvate are also gradually formed. The accumulations of pyruvate and petivericin eventually inhibit the activity of the alliinase. The gradual inactivation of the alliinase by these products interrupts the continuous production of the PMTSO, thiosulfates, and other cytotoxic downstream products such as various oligosulfides.

mixture, as would be the case under physiological conditions, the inhibitory effect was enhanced.

DISCUSSION

We demonstrate here not only how DART-HRMS can be used to monitor highly labile organosulfur plant chemical defense compounds, but also how they can be quantified by DART-HRMS to reveal feedback-inhibition phenomena such as that illustrated in this work. In the aggregate, the results confirm the presence of a negative feedback loop by which the action of the *P. alliacea* alliinase in the production of reactive downstream organosulfur compounds such as the thiosulfinate is stymied shortly after its activation (Scheme 2).⁴⁵ Upon tissue breach, exposure of petiveriin to alliinase results in alliinase-mediated formation of the corresponding transient phenylmethanesulfinic acid (PMSA, Scheme 2).¹³ PMSA, which serves as both the substrate for the LFS and the precursor to petivericin,^{20,46} is sequestered by the LFS, which in turn catalyzes the explosive formation of the lachrymator PMTSO.⁴⁷ We propose that this reaction sequence functions as a first line of defense and has the advantage of immediately deterring vertebrate herbivores by inducing tearing and painful irritation of the eyes, nose, and lungs that lasts several hours.¹² The siphoning off of PMSA by the LFS at the expense of petivericin formation ensures that, at the start, large amounts of PMTSO can immediately be deployed prior to the generation of concentrations of petivericin that are significant enough to curtail PMSA production (and, by extension, lachrymator production) by the alliinase.¹² Because pyruvate and petivericin inhibit the alliinase to varying degrees, the gradual increase in their concentrations eventually results in a significant reduction in

alliinase activity. Importantly, the shutting down of alliinase also curtails production of the lachrymator, because the alliinase provides the substrate (i.e., the sulfinic acid PMSA) that the LFS converts to the lachrymator.

A second element of the defense strategy involves the antimicrobial effects exhibited by the very thiosulfinate that features prominently in the negative feedback loop. Petivericin and other naturally occurring thiosulfonates, such as allacin found in garlic and the 1-propenyl/methyl/propyl thiosulfonates found in onion, have been shown to exhibit a broad spectrum of antibacterial, antifungal, and antiviral activity at concentrations that are attained in planta.^{48–51} The deployment of antimicrobial thiosulfonates and the herbivore deterrent PMTSO occur within seconds of plant wounding, whereas the inhibition of alliinase activity that occurs with increasing concentrations of pyruvate and petivericin occurs ~1–2 h postwounding. Thus, the thiosulfinate produced serves a dual role composed of both antimicrobial and alliinase inhibitory activities.

Our observations indicate that one short-term mechanism by which plants that exhibit this defense chemistry can curtail the negative impact of a tissue breach is through the rapid deployment of compounds such as the lachrymator and thiosulfinate produced in *P. alliacea*. Although these compounds are corrosive, the ability of the thiosulfinate to ultimately inhibit the activity of the enzyme that initiates the cascade of reactions that result in their formation (i.e., alliinase) ensures reduced collateral damage. Our observations also may shed light on the heretofore unexplained finding in studies of *Alliums* and other plants such as onion (*Allium cepa*),^{52–55} garlic (*Allium sativum*),^{56,57} *Tulbaghia violacea*,⁵⁶

and *Albizzia lophantha*⁵⁸) that alliinases appear to be inactivated before all of the available cysteine sulfoxide substrate has been exhausted. Similar to what we observed in *P. alliacea*, the alliinases in these plants may be shut down shortly after their activation, by the thiosulfates that are formed when there is a tissue breach, and this would result in much of the cysteine sulfoxide substrate remaining unreacted.

The nature of the enhanced sensitivity of the alliinase to the effects of petivericin, as well as the possible impact of various other downstream small-molecule degradation products such as SO₂ and elemental sulfur on alliinase activity, are the subjects of continuing investigations in our laboratory. DART-HRMS was well-suited to the investigations described here because of (1) the ability to detect labile organosulfur compounds without causing their degradation or transformation to other species; (2) the ability to detect the compounds of interest at the same time within the same experiment; and (3) the ability to allow quantification of the compounds of interest through the added use of isotopically labeled internal standards. PTR-MS has also been shown to be similarly well-suited to detection of organosulfur natural products. For example, it has been used in combination with isotopic labeling to study sulfur compounds in livestock malodorous manure.⁵⁹ The utilization of milder approaches such as DART-HRMS for the study of organosulfur compounds in particular has been highlighted by Block, who has demonstrated several examples of erroneous conclusions that have been advanced in earlier studies based on the detection of method-induced artifact formation.^{1,60} However, it is worth noting that the acquisition of the consistent results that enabled quantification by DART-HRMS, particularly when using the DIP-it tips and linear rail system for semiautomation, required that due attention was given to the sampling protocol. The following were found: (1) A linear rail speed of ≤ 1 mm/s prevented sample carryover and enabled baseline separation of the peaks in the total ion chromatograms. (2) For the analytes of interest, the application of approximately ≥ 0.8 ng of sample to each DIP-it tip was required to generate a strong DART-HRMS signal. (3) It was essential that the solvent from the solution that was applied to the DIP-it tips be allowed to dry prior to DART-HRMS analysis, because when the sample is in liquid form, it wraps around the DIP-it tips as they enter the DART gas stream, which results in inconsistent amounts of sample being ionized and detected. (4) It is essential that the sample be applied to the same point on all of the DIP-it tubes and that the tubes be mounted in the linear rail such that the samples on each tube are each similarly exposed to the DART gas stream. In this work, this was accomplished by applying the samples to the tips of the DIP-it tubes, and visual verification ensured that the tubes in the linear rail were mounted at the same height.

It remains to be seen whether species that produce lachrymators of a chemical class different from sulfines (such as *Brassica* species plants that produce isothiocyanate lachrymators) utilize a similar negative feedback loop to protect cells at the site of tissue injury. The possibility that other downstream small molecules influence alliinase activity, and the mechanism of the negative feedback inhibition, are under investigation in our laboratory.

CONCLUSIONS

DART-HRMS can serve as a powerful tool for the detection and tracking of induced and labile organosulfur defense

compounds that might otherwise be degraded by other harsher conventional methods. The application of this technique for the molecule tracking, and quantification of such compounds using isotopically labeled analogues, revealed a novel negative feedback inhibition mechanism whereby products downstream of an alliinase can reduce alliinase activity. The results provide a framework within which heretofore unexplained phenomena observed in the defense chemistry of *P. alliacea*, onion, garlic, and other plants can be explained. The method described here also illustrates how the tracking of labile organosulfur natural products can be coupled to their quantification by DART-HRMS using deuterated internal standards. The approach can be used to study enzyme kinetics in plant materials and plant defense chemistry.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.8b03295.

Synthesis protocol and compound characterization for the deuterated internal standard petivericin-*d*₁₄ (i.e., *S*-benzyl phenylmethanethiosulfinate-*d*₁₄) used in this study; acquisition of mass spectra by DART-HRMS using DIP-it tips; standard curves for the quantification of the alliinase substrate and the formation of alliinase-mediated reaction products; results of DART-HRMS monitoring of the production of the lachrymatory sulfine over time; and total ion counts and peak area ratios used for creation of the standard curves (along with the associated standard deviations) (PDF)

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Notes

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