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Reglucosylation of the Benzoxazinoid DIMBOA with Inversion of Stereochemical Configuration is a Detoxification Strategy in Lepidopteran Herbivores**

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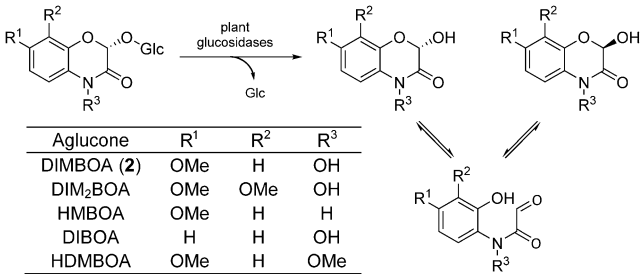
Abstract: *Benzoxazinoids are chemical defenses against herbivores and are produced by many members of the grass family. These compounds are stored as stable glucosides in plant cells and require the activity of glucosidases to release the corresponding toxic aglucones. In maize leaves, the most abundant benzoxazinoid is (2R)-DIMBOA-Glc, which is converted into the toxic DIMBOA upon herbivory. The ways in which three Spodoptera species metabolize this toxin were investigated. (2S)-DIMBOA-Glc, an epimer of the initial plant compound, was observed in the insect frass, and the associated glucosyltransferase activity was detected in the insect gut tissue. The epimeric glucoside produced by the insect was found to be no longer reactive towards plant glucosidases and thus cannot be converted into a toxin. Stereoselective reglucosylation thus represents a detoxification strategy in Spodoptera species that might help to explain their success as agricultural pests on benzoxazinoid-containing crops.*

To avoid damage by insect herbivores, plants have evolved diverse defense mechanisms, including the production of toxic or deterrent metabolites. In response, many insect species have developed adaptations that enable them to feed on chemically-defended plants without apparent negative effects.[1] These adaptations include the rapid excretion of defense compounds,[2] sequestration,[3] and detoxification.[4] Collectively, these adaptations contribute to the unparalleled ecological success of insect herbivores in terrestrial ecosystems. Adaptations to plant defense traits have also favored the emergence of some species as agricultural pests, as is the case with some lepidopteran caterpillars.[5]

Benzoxazinoids (BXDs) are indole-derived plant defense compounds that are widespread in grasses (Poaceae), including crops like wheat, rye, and maize.[6] They are stored as stable glucosides in plant cells and are hydrolyzed to toxic aglucones upon damage, when they come into contact with specific β-glucosidases (Scheme 1).[7] The aglucones formed



Aglucone	R^1	R^2	R^3
DIMBOA (**2**)	OMe	H	OH
DIM_2BOA	OMe	OMe	OH
HMBOA	OMe	H	H
DIBOA	H	H	OH
HDMBOA	OMe	H	OMe

Scheme 1. Benzoxazinoids are plant defense compounds that are activated by plant glucosidases to release toxic aglucones. Different substitution patterns give rise to the many representatives of this class.

are cyclic hemiacetals that form α-oxo aldehydes through ring opening and thereby become reactive towards a wide range of biological nucleophiles.[8] The quantities and proportions of BXDs in grasses vary between different plant species,[6a] organs, and developmental stages,[9] and after induction by herbivores.[10] The most abundant BXD in young maize (*Zea mays*) leaves is (2*R*)-2-β-D-glucopyranosyloxy-4-hydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIMBOA-Glc, **1**). The corresponding aglucone (DIMBOA, **2**) delays growth and decreases survival in several Lepidopteran species, including *Sesamia nonagrioides*,[11] *Ostrinia nubilalis*,[12] *Ostrinia furnacalis*,[13] *Spodoptera littoralis*,[10] and *Spodoptera exigua*,[14] while *Spodoptera frugiperda* seems to be less affected.[10,14] Moreover, **2** inhibits peptidases in *S. nonagrioides*[11] and detoxification enzymes such as glutathione *S*-transferases and esterases in the aphid *Rhopalosiphum padi*.[15] The ability of herbivores to metabolize **2** could thus facilitate their capacity to feed on maize. Although BXD biosynthesis in plants is well-studied,[7] knowledge of their metabolic fate in herbivores is limited and would provide

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information about detoxification mechanisms and their role in host selection.

To compare BXD metabolism among various Lepidoptera, we analyzed frass (caterpillar feces) samples from *S. frugiperda*, *S. littoralis*, *S. exigua*, *Mamestra brassicae*, and *Helicoverpa armigera*, all of which had previously fed on maize leaves. LC–MS/MS with multiple reaction monitoring (MRM) was used to separate and detect BXD glucosides and aglucones (Figure 1). We observed a peak corresponding to

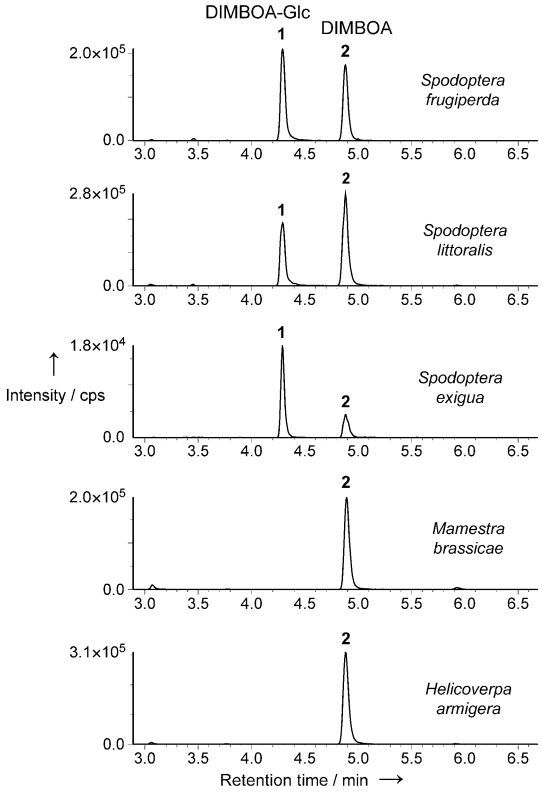


Figure 1. Benzoxazinoid profiles of frass collected from different insect species after they had fed on maize containing DIMBOA-Glc (**1**) demonstrated that this compound is present in *S. frugiperda*, *S. littoralis*, and *S. exigua* after digestion, but not in *M. brassicae* and *H. armigera*. Fourth instar larvae were fed on maize leaves for 48 h. Frass samples were collected, extracted with acidified water/methanol (50:50 v/v, 0.5% formic acid), and analyzed by LC–MS/MS(MRM). Only chromatographic traces for DIMBOA-Glc (**1**) and DIMBOA (**2**) are depicted. The intensities in counts per second (cps) do not reflect quantitative ratios.

glucoside **1** in samples from *S. frugiperda*, *S. littoralis*, and *S. exigua*, but not from *M. brassicae* and *H. armigera*. This result suggests that the former group of species either excretes intact **1** by inhibiting its hydrolysis or reglycosylates **2** during digestion, as previously hypothesized.[10]

To test this putative reglycosylation, we incubated cell-free suspensions from *S. frugiperda* gut tissues with **2** and uridine diphosphate glucose (UDP-glucose). A peak corresponding to **1** confirmed DIMBOA-UDP-glucosyltransferase (UGT) activity (Figure 2A). This activity was highest at pH values around 7.0 (data not shown), thus suggesting the action

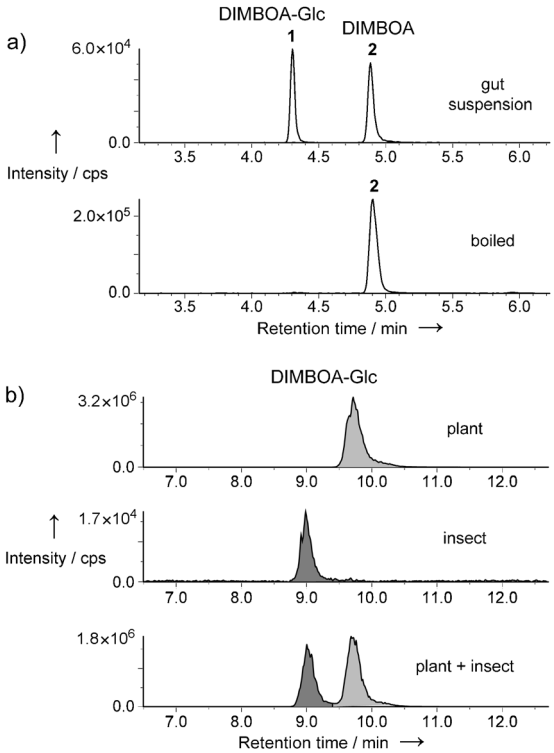


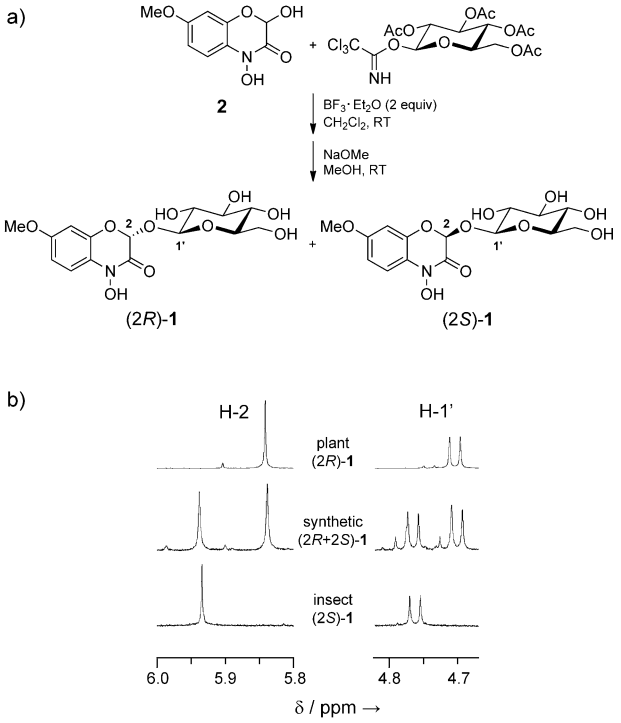
Figure 2. a) Incubation of DIMBOA (**2**) and UDP-glucose with *S. frugiperda* cell-free gut suspensions led to the production of DIMBOA-Glc (**1**; top chromatogram), thus indicating UDP-glucosyltransferase activity that is not observed when using boiled gut preparations (bottom chromatogram). Fourth instar *S. frugiperda* and *S. littoralis* larvae fed either on artificial diet or maize leaves for 48 h were dissected and homogenized, and cell-free gut suspensions were incubated with 3 mM DIMBOA and 6 mM UDP-glucose at pH 4.0, 5.4, 7.0, 8.4, and 10.0 at 30°C for 30 min, followed by the addition of methanol/formic acid (1:1, v/v), centrifugation, and analysis by LC–MS/MS(MRM). Only assays at pH 7.0 with gut extracts from plant-fed *S. frugiperda* are presented. b) Chromatography under optimized conditions revealed that the DIMBOA-Glc observed in insect frass samples (dark gray) is different from the plant DIMBOA-Glc (light gray). Plant and *S. frugiperda* frass samples were extracted with acidified water/methanol (50:50 v/v, 0.5% formic acid) and analyzed by LC–MS/MS(MRM). The intensities in counts per second (cps) do not reflect quantitative ratios.

of an intracellular enzyme, which is in agreement with the membrane association of most insect UGTs.[16]

A close comparison between chromatograms revealed a small difference in retention time between the peaks corresponding to **1** in samples from plant and insect (both frass and in vitro assays). After optimizing HPLC separation, we confirmed that plant- and insect- derived **1** are indeed different compounds (Figure 2B). Since both compounds present similar fragmentation patterns and MS^2 spectra (Figure S1 in the Supporting Information), we hypothesized that they might differ in terms of stereochemical configuration.

In order to elucidate the structure of insect-derived **1**, we purified it from *S. frugiperda* frass extracts and compared its ^1H NMR spectrum (Figure S2) to that of the plant-derived compound (Figure S3). Axial–axial coupling constants of

around 8–9 Hz for the glycoside moiety of the insect compound confirmed that it is also a β-glucoside. However, large differences in the chemical shifts for H-2 and H-1′ (Table S1 in the Supporting Information) suggested that plant- and insect-derived **1** may differ in their configuration at C-2. Grasses exclusively accumulate (2*R*)-DIMBOA-Glc [(2*R*)-**1**],[17] which can be synthesized in a diastereoselective fashion.[18] By modifying this published synthetic method, we chemically glucosylated DIMBOA to yield both (2*R*)- and (2*S*)-**1** epimers in a 1:1 ratio (Scheme 2 A). After comparing the NMR spectra from plant-derived, insect-derived, and synthetic samples of **1** (Scheme 2 B), we were able to determine that the insect-derived compound is in fact (2*S*)-DIMBOA-Glc [(2*S*)-**1**].



Scheme 2. a) Chemical glucosylation of DIMBOA (**2**) to yield (2*R*)- and (2*S*)-DIMBOA-Glc (**1**) in a 1:1 ratio by using a method modified from Kluge and Sicker;[18] b) A comparison of partial ^{1}H NMR (500.13 MHz, [D$_6$]acetone) spectra from **1** obtained from the plant, insect frass, and chemical synthesis demonstrates that the insect-derived compound corresponds to (2*S*)-**1**, an epimer of the plant compound.

Since we exclusively observed the (2*S*)-**1** epimer in frass samples, we assessed its abundance relative to (2*R*)-**1** during the course of digestion in the insect gut. We dissected maize-fed insects and analyzed the contents of different regions of the gut by LC–MS/MS. There was a gradual increase in the ratio of (2*S*)-**1** to (2*R*)-**1** upon progression through the foregut and midgut (Figure 3). This ratio markedly increased in hindgut/rectum, and (2*S*)-**1** was the exclusive form excreted in the frass. Furthermore, we observed (2*S*)-**1**, but not (2*R*)-**1** or **2**, in hemolymph from insects feeding on maize leaves (data not shown).

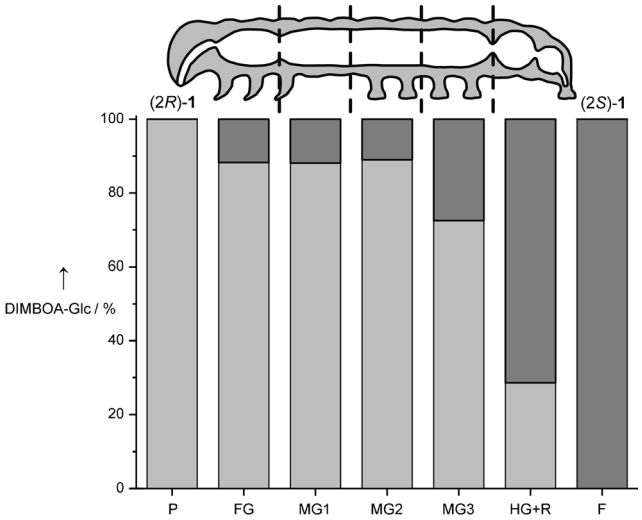


Figure 3. LC–MS analyses of the contents of different gut regions evidence a gradual conversion of (2*R*)-DIMBOA-Glc (**1**; light gray) into (2*S*)-**1** (dark gray) along the course of the insect gut (P plant, FG foregut, MG midgut regions, HG + R hindgut/rectum, F frass). Guts of fourth instar *S. frugiperda* fed on maize leaves for 4 days were dissected and separated into five regions, the contents of which were individually collected, extracted with acidified water/methanol (50:50 v/v, 0.5% formic acid), and analyzed by LC–MS/MS(MRM). Bars represent peak areas for (2*R*)-**1** and (2*S*)-**1** normalized to their sum. The results from one experiment are shown but three replicates showed the same general profile.

Simple reglucosylation to afford a BXD glucoside could be reversed by plant glucosidases, which are likely active along the entire course of the insect gut.[10] This would lead to energy expenditure by the insect instead of effective detoxification. We examined whether the change from (2*R*)-**1** to (2*S*)-**1** affects its suitability as a glucosidase substrate. Insects fed on maize leaves were dissected and the gut contents (containing both epimers of **1** and glucosidases) were incubated in vitro at different pH values. Plant glucosidases were active even after going through the digestion process and displayed striking specificity towards the plant-produced (2*R*)-**1**, whereas the insect-derived (2*S*)-**1** remained unhydrolyzed (Figure 4). Although this glucosidase activity was higher at pH 7.0, it was easily detected after 30 min at pH 10.0. These incubation conditions are realistic considering that lepidopteran guts are highly alkaline[19] and the digestion of plant material averages several hours.[20] In *S. frugiperda*, we measured pH values ranging from 8.5 to 9.2 in all regions of the gut, except in the rectum, where near-neutrality was observed (Figure S4). The glucosidase activity observed at these pH values fits with the corresponding ratios of (2*S*)-**1** to (2*R*)-**1** observed through the gut, and further corroborates previous observations that BXD aglucones are constantly released along the course of the insect gut during digestion.[10] Therefore, the insect UGT not only stabilizes the reactive **2** by glucosylation, but its stereoselectivity additionally renders the new glucoside (2*S*)-**1** inert towards the still-active plant glucosidases, thereby preventing its further hydrolysis to restore **2**.

Since insect UGTs are intracellular and typically membrane-associated, we hypothesize that the reglucosylation of **2**

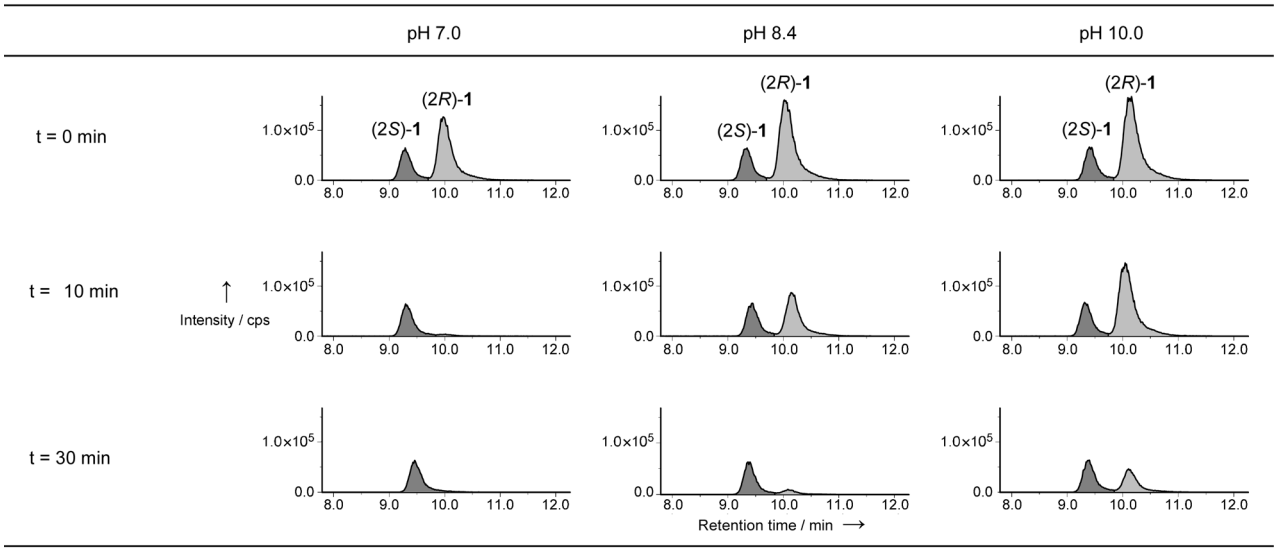


Figure 4. Incubation of the gut contents of maize-fed *S. littoralis*, which contain the plant DIMBOA-Glc (2*R*)-**1** and the insect metabolite (2*S*)-**1**, at different pH values indicates that plant glucosidases are still active under all conditions tested and are specific towards (2*R*)-**1** (light gray). The insect-derived (2*S*)-**1** (dark gray) was not hydrolyzed. Fourth instar *S. littoralis* larvae fed on maize leaves for 8 h were dissected and the total gut contents, which naturally contain both (2*S*)- and (2*R*)-**1**, were incubated at pH 7.0, 8.4, and 10.0 at 30 °C for 10 or 30 min, followed by the addition of methanol/formic acid (1:1, v/v), centrifugation, and analysis by LC–MS/MS(MRM).

takes place inside the gut cells after absorption. As an aglucone, **2** is quickly converted into a racemic mixture by oxo-cyclo-tautomerism[21] and is taken up by the insect UDP-glucosyltransferase, which displays a higher affinity for the aglucone substrate that leads to formation of the 2*S* product. A dynamic kinetic resolution of **2** is thus established through its stereoselective conversion to (2*S*)-**1**. The resulting biologically inert (2*S*)-**1** may be actively transported 1) back to the gut contents and/or 2) to the hemolymph, from which it can be delivered into the hindgut contents later. Subsequently, (2*S*)-**1** is excreted and represents the only epimer of DIMBOA-Glc in the frass.

Our results indicate that the stereoselective reglucosylation of DIMBOA to (2*S*)-DIMBOA-Glc is a detoxification mechanism in the *Spodoptera* species we studied. DIMBOA-UGT activity in *Spodoptera* and other Lepidoptera has already been demonstrated or suggested.[10,22] Our work provides information about the stereoselectivity of this transformation. This strategy may be widespread in the genus *Spodoptera*, which would be consistent with the elevated degree of tolerance towards maize and BXDs in this genus.[10] Glucosylation is an important detoxification pathway that stabilizes toxins and favors excretion.[23,24] To our knowledge, the epimerization of plant defensive compounds by insects has only been described so far for butterflies that use plant pyrrolizidine alkaloids as precursors for pheromone biosynthesis.[25] In this context, our work describes a novel insect herbivore detoxification strategy that is based on the stereochemical inversion of one chiral center to stabilize and deactivate a plant defensive compound. Future characterization of the insect UGT enzyme(s) involved in DIMBOA detoxification should shed light on its efficiency, specificity, evolutionary origin, and value to herbivores feeding on maize. This information and further progress in clarifying how lepidopteran herbivores metabolize BXDs should also increase our understanding of their coevolution with grasses.

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