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Taste detection of the non-volatile isothiocyanate moringin results in deterrence to glucosinolate-adapted insect larvae

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ABSTRACT

Isothiocyanates (ITCs), released from Brassicales plants after hydrolysis of glucosinolates, are known for their negative effects on herbivores but mechanisms have been elusive. The ITCs are initially present in dissolved form at the site of herbivore feeding, but volatile ITCs may subsequently enter the gas phase and all ITCs may react with matrix components. Deterrence to herbivores resulting from topically applied volatile ITCs in artificial feeding assays may hence lead to ambiguous conclusions. In the present study, the non-volatile ITC moringin (4-(α-L-rhamnopyranosyloxy)benzyl ITC) and its glucosinolate precursor glucomoringin were examined for effects on behaviour and taste physiology of specialist insect herbivores of Brassicales. In feeding bioassays, glucomoringin was not deterrent to larvae of *Pieris napi* (Lepidoptera: Pieridae) and *Athalia rosae* (Hymenoptera: Tenthredinidae), which are adapted to glucosinolates. Glucomoringin stimulated feeding of larvae of the related *Pieris brassicae* (Lepidoptera: Pieridae) and also elicited electrophysiological activity from a glucosinolate-sensitive gustatory neuron in the lateral maxillary taste sensilla. In contrast, the ITC moringin was deterrent to *P. napi* and *P. brassicae* at high levels and to *A. rosae* at both high and low levels when topically applied to cabbage leaf discs (either 12, 120 or 1200 nmol moringin per leaf disc of 1 cm diameter). Survival of *A. rosae* was also significantly reduced when larvae were kept on leaves treated with moringin for several days. Furthermore, moringin elicited electrophysiological activity in a deterrent-sensitive neuron in the medial maxillary taste sensillum of *P. brassicae*, providing a sensory mechanism for the deterrence and the first known ITC taste response of an insect. In simulated feeding assays, recovery of moringin was high, in accordance with its non-volatile nature. Our results demonstrate taste-mediated deterrence of a non-volatile, natural ITC to glucosinolate-adapted insects.

1. Introduction

Glucosinolates are defensive chemicals in the plant order Brassicales. Upon tissue disruption they are converted to isothiocyanates (ITCs) or other products due to endogenous enzymes, the myrosinases (β-thioglucoside glucohydrolases; E.C. 3.2.1.147) (Agerbirk and Olsen, 2012). The ITCs, commonly known as mustard oils, are reactive, pungent chemicals. Hence, the glucosinolate–myrosinase system has been aptly named the mustard oil bomb (Matile, 1980). While the mustard oil bomb is an effective defense against many herbivores, a number of Brassicales specialist insects can usually avoid ITC formation in the gut thanks to various biochemical mechanisms (Müller, 2009; Winde and Wittstock, 2011; Opitz et al., 2011; Beran et al., 2014).

A chewing herbivore will initially come into contact with an ITC in the water-dissolved form, and dissolved ITC concentrations could serve to guide its feeding response. Only subsequently, volatile ITCs will enter the gas phase. Remarkably, excitation of a taste neuron by an ITC has never been demonstrated. In contrast, in insect-plant research, a few volatile (commercially available) ITCs have mainly been used for behavioural studies and olfactory research (Li et al., 2000; Agrawal and Kurashige, 2003; Barker et al., 2006). However, much uncertainty regarding suitable methods for toxicity and deterrency testing of ITCs is apparent from the literature. First, volatile

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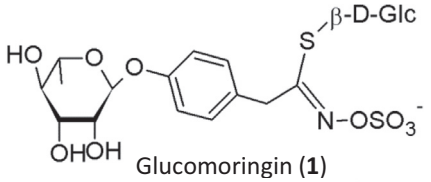
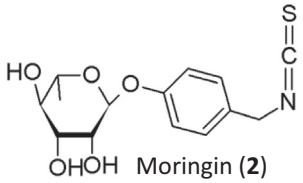
ITCs quickly evaporate, making the actual concentrations present in bioassays difficult to estimate. Second, chemical reactions with matrix components based on addition of ITCs to various substrates can occur in these bioassays which typically last hours or longer.

In a pioneering study on ITC effects on insects, allyl ITC was added to a synthetic diet offered to a glucosinolate-adapted moth, and LC_{50} values in the low μmol/g diet fresh wt. range were reported (Li et al., 2000). In a later paper (Agrawal and Kurashige, 2003), it was argued that studies employing addition of ITCs to synthetic diets had failed because allyl ITC is volatile and will be released to fumigate the larvae. Using microencapsulated allyl ITC molecules in synthetic diets, a toxic effect on larvae of a glucosinolate-adapted butterfly was demonstrated, with LC_{50} around 2 μmol/g diet fresh wt. (as judged from presented graphs) (Agrawal and Kurashige, 2003). A more recent approach employed dilute solutions of benzyl ITC sprayed every 48 h on test leaves offered to caterpillars (Bejai et al., 2012). In this study, evaporation of the ITC was suggested to be prevented by using closed Petri dishes sealed with cellophane tape. However, levels of benzyl ITC on the test leaves (and in the atmosphere in the Petri dishes) were not experimentally controlled. From all three reports (Li et al., 2000; Agrawal and Kurashige, 2003; Bejai et al., 2012) it is evident that an experimental measurement of the actual levels of ITCs during biological testing is needed for quantitative conclusions, and that both volatility and reactivity of the ITC should be considered. A non-volatile ITC offers a useful opportunity for testing whether ITCs as such in the liquid phase are deterrent and/or growth-inhibiting to insect larvae and if they are perceived at the level of taste neurons.

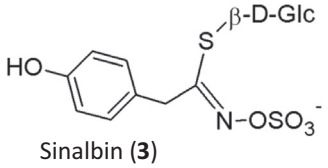
We expected the rare class of side chain glycosylated glucosinolates (Kjær et al., 1979; Olsen and Sørensen, 1979; Olsen et al., 1981; Gueyrard et al., 2000; Bennett et al., 2003; Kim et al., 2004) to form particularly non-volatile ITCs. A representative glucosinolate from this structural class is 4-(α-L-rhamnopyranosyloxy)benzylglucosinolate (**1**) (Kjær et al., 1979; Gueyrard et al., 2000), which is traditionally known from trees of the tropical genus *Moringa* but was more recently discovered in herbs from temperate climates (de Graaf et al., 2015). The common name glucomoringin was recently suggested for this compound (Brunelli et al., 2010), in accordance with the principles of classical glucosinolate nomenclature (Kjær, 1960). According to the same nomenclature, the corresponding ITC (4-(α-L-rhamnopyranosyloxy) benzyl ITC) (**2**) would be named moringin. Recently, antibiotic properties as well as promising physiological activities in mammals have been reported for moringin (Galuppo et al., 2013; Waterman et al., 2014; Giacoppo et al., 2015). However, effects of side chain glycosylated glucosinolates and their ITCs on insect herbivores have to our knowledge so far not been investigated.



Glucomoringin (**1**)



Moringin (**2**)



Sinalbin (**3**)

The aims of this work were (1) to isolate and characterize a side chain glycosylated glucosinolate and its corresponding natural, non-volatile ITC, (2) to test the effects of these compounds on performance, feeding preference and taste physiology of glucosinolate-adapted insect larvae, and (3) to critically examine the recovery of the ITC under feeding assay conditions. As glucosinolate-adapted insects, we chose larvae of the model insects *Pieris napi* and *Pieris brassicae* (Lepidoptera: Pieridae) and *Athalia rosae* (Hymenoptera: Tenthredinidae), that represent two different mustard oil bomb avoidance strategies, metabolic diversion in *Pieris* spp. (Wittstock et al., 2004; Agerbirk et al., 2006, 2010b; Stauber et al., 2012) and glucosinolate sequestration with desulfation/sulfation in *A. rosae* (Müller et al., 2001; Opitz et al., 2011; Abdalsamee et al., 2014), respectively. *P. brassicae* was included since it is particularly amenable to electrophysiological experiments (Schoonhoven and van Loon, 2002). For comparison in some experiments, we included a glucosinolate without the critical glycosylation, *p*-hydroxybenzylglucosinolate (sinalbin) (**3**). We demonstrate that the non-volatile ITC moringin is a suitable model to test taste-mediated ITC effects on insects due to its lack of volatility, stimulation of a taste neuron, deterrent effects and high recovery in bioassays.

2. Results and discussion

2.1. Isolation and characterization of glucomoringin, moringin and desulfoglucomoringin

Glucomoringin (**1**) and moringin (**2**) were isolated at the Bologna laboratory (CRA-CIN) from *Moringa oleifera* seeds using established methods (Section 3.2), and their structures confirmed by one and two-dimensional NMR spectroscopic analyses (Sections 3.8 and 3.9). The preparations were essentially pure as judged from ^{1}H NMR spectra and HPLC chromatograms (Section 3.2). The chemical shifts essentially agreed with previously published data (Gueyrard et al., 2010; de Graaf et al., 2015). For moringin, however, a possible moderate difference of the chemical shift of the quaternary ITC carbon was observed as compared to a recent report using a different solvent (de Graaf et al., 2015). The signal was very weak as expected for an ITC (Glaser et al., 2015), and was not detected in the one-dimensional ^{13}C NMR spectrum. The value reported here (133 ppm extracted from the HMBC spectrum due to correlation with the methylene H) agreed with the calculated value using a standard NMR prediction tool (www.nmrdb.org). The L-configuration of the rhamnose residue could not be concluded from NMR but was known from a published comparison of the *M. oleifera* compound with an authentic, synthetic standard (Gueyrard et al., 2000).

The ion trap mass spectrum of glucomoringin after analytical desulfation was of interest because the analytical chemistry of side chain glycosylated glucosinolates is poorly known. It was measured along with control spectra of desulfoderivatives of related glucosinolates, namely benzylglucosinolate, its *p*-hydroxyl derivative **3** and the *p*-methoxyl derivative (glucoaubrietin) (Fig. S1). These controls served to evaluate the structural basis of specific cleavages observed for desulfo glucomoringin. The electrospray ionization with NaCl-spiked eluents produced solely sodium adducts, whereas proton adducts were not observed. The ion trap mass spectra of the three control glucosinolates provided evidence of very similar fragmentation as reported for many other glucosinolates (Agerbirk et al., 2014, 2015). They were dominated by sodium adducts of thioGlc (base peak, *m*/*z* 219) and anhydroGlc (*m*/*z* 185) while the "type c" adduct of the aglucone after loss of anhydroGlc was less abundant (16–25% of base peak) (Fig. S1). The ion trap mass spectrum of the desulfo glucomoringin Na^+

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