Chemoecology (2014) 24:35–39
DOI 10.1007/s00049-013-0144-z

CHEMOECOLOGY

SHORT COMMUNICATION

Stereochemistry affects sesquiterpene lactone bioactivity against an herbivorous grasshopper

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Received: 21 September 2013 / Accepted: 10 December 2013 / Published online: 24 December 2013

Abstract Sesquiterpene lactones are defensive compounds which protect plants against a variety of herbivores and other natural enemies. Sesquiterpene lactones from higher plants can be divided into two groups based on the stereochemistry of their lactone ring junction, either *cis*-fused or *trans*-fused. It is unclear whether and how this variation affects potentially important ecological interactions. To investigate whether stereochemical variation in sesquiterpene lactone ring junctions can influence resistance to herbivorous insects, we performed controlled feeding trials with two pairs of diastereomeric sesquiterpene lactones and examined the deterrent effect of each compound on feeding by the polyphagous grasshopper *Schistocerca americana* (Drury). Sesquiterpene lactone stereochemistry and concentration significantly influenced feeding behavior with grasshoppers consuming less of the *trans*-fused compounds than the *cis*-fused compounds. To our knowledge, this is the first demonstration that sesquiterpene lactone ring junction stereochemistry influences the feeding behavior of herbivores. Because this stereochemical trait polymorphism is widely distributed in nature, it could have substantial consequences for the ecology and evolution of large groups of plants, particularly the Asteraceae.

Keywords *Xanthium strumarium* · Isomer · Herbivory · Secondary metabolite · Plant defense · Feeding trial

Handling Editor: Paulo H. G. Zarbin.

Electronic supplementary material The online version of this article (doi:10.1007/s00049-013-0144-z) contains supplementary material, which is available to authorized users.

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Introduction

Sesquiterpene lactones (STL) are an exceptionally diverse class of plant secondary metabolites with close to 5,000 structures elucidated (Harborne et al. 1999; Schmidt 2006). They are believed to serve as defensive compounds in plants with lab assays showing activity (typically mediated by the lactone ring moiety) against bacteria, fungi, mollusks, insects, and mammals (Rodriguez et al. 1976; Picman 1986; Schmidt 1999). In insects they have been shown to function as feeding deterrents and developmental inhibitors, and to reduce survival (Picman 1986). Sesquiterpene lactones have a broad phylogenetic distribution in plants, but are particularly diverse and abundant in the Asteraceae (Yoshioka et al. 1973), leading some to speculate that STL have contributed to the evolutionary success of this exceptionally large group (Seaman 1982; Schmidt 1999).

Stereochemical variation is one understudied feature that could potentially have a profound effect on the activity and ecological effects of STL (Schmidt 2006). Across plants, there are numerous examples of compounds differing solely in the stereochemistry of their lactone ring junction (see Budesínský and Saman 1995). In vitro laboratory assays with diastereomeric STL have indicated that

stereochemistry can influence activity (see Supplemental Material for a brief review), but in vivo tests are lacking, as are tests using ecologically relevant organisms (i.e., those that might have shaped the evolution of such compounds in nature) such as herbivores. Here, we ask: does the stereochemistry of the lactone ring junction of STL influence the feeding behavior of an herbivorous grasshopper? We isolated two pairs of diastereomeric STL from the plant *Xanthium strumarium* L. (Asteraceae). We then conducted no-choice feeding experiments with the polyphagous grasshopper *Schistocerca americana*, examining (1) whether the tested STL deterred feeding and (2) if diastereomers differed in their deterrent effect.

Methods

Here we outline the experimental protocol; full methods are presented in the Supplemental Material.

Test compound isolation and structural identification

In order to investigate the effects of STL ring junction stereochemistry on feeding deterrence, we first set out to isolate diastereomeric pairs of STL differing solely in the stereochemistry of the lactone ring junction. Two pairs of diastereomeric sesquiterpene lactones (see Fig. 1) differing in the bond geometry of a single carbon were extracted from aerial portions of *Xanthium strumarium* plants. Plants were collected from two natural populations, one with *cis*-fused (Liberty, TX) and one with *trans*-fused (Nixon, TX) lactone rings. Although a number of STL were present in each plant type, we selected the two pairs used because they were able to be isolated in sufficient quantities for bioassays. All steps in the isolation process were monitored via thin layer chromatography (TLC). Methylene chloride extracts were filtered, concentrated, and separated into crude fractions via dry column vacuum chromatography (Harwood 1985). Compounds were then separated via flash chromatography. All STL were identified by ^{1}H and 2D NOESY experiments on a Varian Inova 600 MHz NMR. Compounds were identified by comparison with published ^{1}H NMR data of STL isolated from *Xanthium strumarium*. To verify lactone junction stereochemistry for each compound, we performed additional NOESY experiments and made stereochemical assignments based on NOE's between H-8 and adjacent protons (Figure S1).

Grasshoppers

Schistocerca americana (Drury) feeds on a wide range of plants in the southeastern US, including many Asteraceae (Otte 1975). Further justification for the choice of this species is given in Supplemental Material (Methods). All grasshoppers used in experiments were reared from eggs from a standing laboratory colony maintained since 2006 by S. Behmer, Texas A&M University (Boswell et al. 2008). Eggs were hatched and nymphs maintained in an insectary in Bioquip cages (61 × 61 × 61 cm) at 31 °C, and photophase L:D 15:9. Newly hatched nymphs were fed *ad libitum* on a mixed diet of fresh wheatgrass seedlings and dry wheat germ throughout their entire developmental period. All experiments were conducted with 1- to 2-day-old 6th instar grasshoppers.

Bioassay protocol

To assess the influence of lactone junction stereochemistry on feeding deterrence in *S. americana*, we conducted no-choice feeding experiments with individual STL applied to sucrose-impregnated glass microfiber discs (Whatman GF/A, 2.1 cm diameter, containing 2.5 % dry weight sucrose as a feeding stimulant). Grasshoppers will readily consume these glass microfiber discs, which are commonly used in grasshopper feeding assays (see Supplemental material and references therein). Each compound was tested across a range of five concentrations, constituting 1, 2, 3, 4, or 5 % dry weight of the disc. These concentrations are biologically relevant, as STL can constitute up to 5 % of plant dry weight (see Supplemental Material). A control treatment (discs with sucrose but no STL) was included.

Each test arena consisted of a 17 × 12 × 6.5 cm ($L \times W \times H$) clear plastic box with ventilation holes in the top. The test filter disc was mounted on an inverted pushpin placed 6.5 and 4 cm from the sides of the box so that the disc was readily accessible to the grasshopper. The other side of each box contained a water source (plastic portion cup with a cotton wick), and a perch made of metal hardware cloth. All boxes were arranged on a table in a testing room maintained at 31 °C, illuminated with overhead incandescent light, with cardboard partitions between boxes to prevent visual interactions between neighboring grasshoppers.

On the morning of each trial day, 1- to 2-day-old 6th instar grasshoppers were selected, starved for 2 h, and introduced individually to test arenas containing a single test filter disc. After 7 h, grasshoppers were removed, starved for 12 h to remove gut contents, and weighed. Due to space limitations, we examined feeding responses to each pair of diastereomers in separate trials, and conducted each trial over a four consecutive days (totaling 8 days).

Discs were weighed after each trial and the percentage mass removed was calculated by dividing by the initial weight of each disc. This response variable was relativized in each trial by dividing the percentage consumed of each disc by the average consumed across control discs (78.4 %

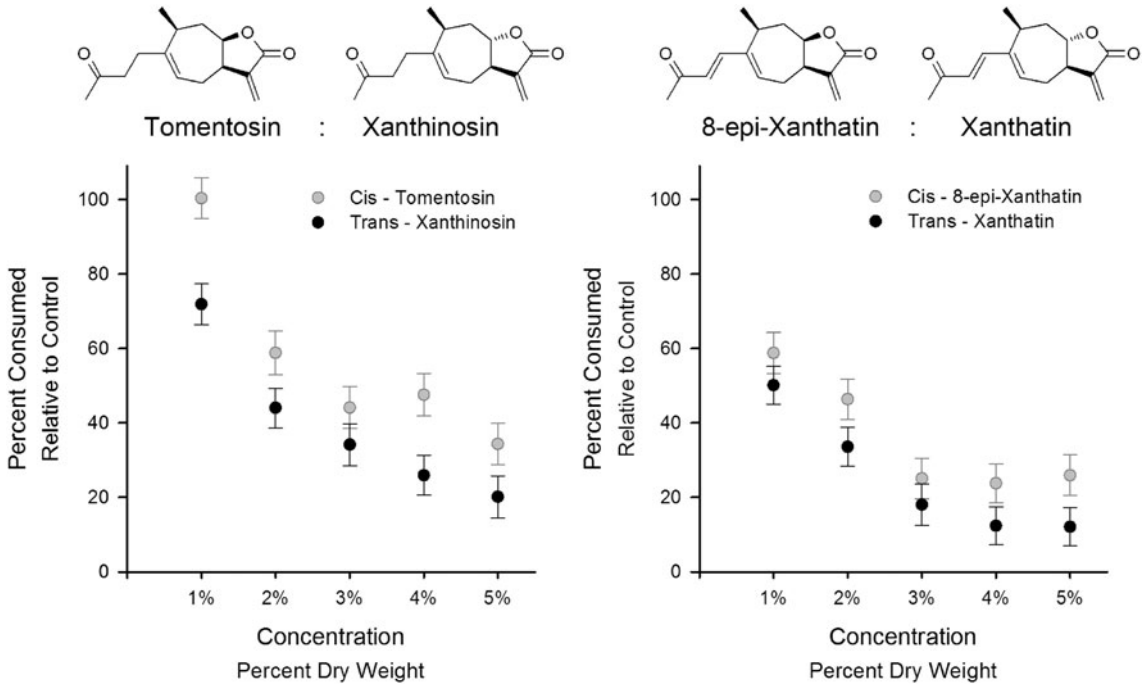

Fig. 1 Percentage consumption of discs by *Schistocerca americana* grasshoppers in response to sesquiterpene lactone stereochemistry and percentage dry weight concentration for two diastereomeric pairs. Consumption measures for test discs were relativized to the consumption of control discs. Least squares means and standard errors are presented for each treatment combination. *Cis*-fused STL are presented in *gray*, *trans*-fused STL are presented in *black*. In both trials, both stereochemistry and concentration significantly influenced feeding consumption by grasshoppers. Least squares mean consumption of discs containing each compound: tomentosin 57.04 %; xanthinosin 39.23 %; 8-epi-xanthatin 35.99 %; xanthatin 25.25 %

for the tomentosin: xanthinosin trial, 71.6 % for the 8-epi-xanthatin: xanthatin trial).

Statistical analysis

Because diastereomer pairs were examined in separate trials, each pair was analyzed in separate statistical models and trial day was incorporated into each model as a covariate. For each pair of compounds, we fit a ANCOVA model using Proc GLM in SAS (SAS Institute 2010), with stereochemistry (either *cis* or *trans*; a categorical fixed effect), concentration (continuous fixed), trial date (categorical fixed), grasshopper weight (continuous fixed), and the stereochemistry × concentration interaction as predictor variables. The stereochemistry × concentration interaction did not improve model fit and was dropped from the final model. We compared the least squares means of the response (percentage of control consumption) for each compound to determine whether there was an overall effect of stereochemistry.

Results

All tested STL reduced feeding by *S. americana*. Furthermore, STL concentration and stereochemistry both significantly influenced consumption (Fig. 1), with *trans*-fused lactone junctions reducing consumption by an average of 33.44 % (range 32.6–34.3 %) relative to *cis*-fused junctions. For the diastereomeric pair tomentosin: xanthinosin, consumption was reduced as concentration increased ($F_{1,188} = 107.04$, $P < 0.001$), and grasshoppers consumed more from discs treated with tomentosin (*cis*) than xanthinosin (*trans*) ($F_{1,188} = 24.56$, $P < 0.001$). Similarly, for the pair 8-epi-xanthatin: xanthatin, consumption was reduced as concentration increased ($F_{1,197} = 61.15$, $P < 0.001$), and grasshoppers consumed more from discs treated with 8-epi-xanthatin (*cis*) than xanthatin (*trans*) ($F_{1,197} = 9.57$, $P = 0.002$). The absence of significant interactive effects between stereochemistry and concentration indicated that the dose-dependency of the grasshopper response was similar for *cis*- and *trans*-fused STL.

Discussion

Our work represents the first empirical evidence that naturally occurring variation in STL stereochemistry influences plant resistance to herbivores (see the Supplemental Material for a review of earlier studies). While consequences for herbivore performance remain unknown,

field work in the *Xanthium* system has demonstrated that this variation in lactone ring stereochemistry translates into significant differences in plant performance in nature, with plants containing *trans*-fused STL often showing lower attack rates and higher fitness (seed production) than their *cis*-fused counterparts (Ahern and Whitney, in press). Thus, stereochemical variation in STL may be an important, understudied factor mediating plant–herbivore interactions.

The importance of stereochemistry in mediating interactions between molecules in biological systems is becoming widely accepted (Testa et al. 2013). Because stereoisomers differ in three-dimensional shapes, they can differ in their ability to access and interact with active sites of many biomolecules, including DNA, enzymes, and receptors. Additionally, members of groups of diastereomeric compounds, such as the STL examined in this study, often differ in physiochemical properties such as lipophilicity, conformational flexibility, and chemical reactivity (Kalsi 2009). Differential activity between stereoisomers could result from the above-mentioned factors, either in isolation or in combination.

Of course, understanding the mechanistic basis of the stereoselective differences in STL effects seen here will not be possible without first identifying specific molecular target(s). This is a potentially a daunting task, because STL have been shown to interfere with a variety of cellular processes and molecular targets (reviewed in Schmidt 1999). However, one potential molecular target is the set of γ-aminobutyric acid (GABA)/glycine sensitive taste receptors. The GABA/glycine taste receptor hypothesis proposes that GABA/glycine sensitive receptors in insect chemosensilla mediate the perception of both feeding stimulants and deterrents (Mullin et al. 1994). Feeding inhibition occurs via the chemical disruption of the normal functioning of neurons which perceive phagostimulants (Mullin et al. 1994).

Two separate lines of evidence suggest that the observed differences in feeding deterrence between *cis*- and *trans*-fused STL could be related to GABA/glycine sensitive receptors. First, the feeding deterrent effects of both STL and the known GABA-antagonists strychnine and picrotoxinin can be negated by simultaneous co-administration of equimolar GABA, indicating that STL act on GABA/glycine sensitive receptors (Passreiter and Isman 1997). Second, GABA receptors have been shown to be highly stereoselective (Krogsgaard-Larsen 1998). Results from feeding deterrence assays with the stereoisomeric GABA antagonist model compounds (+)-bicuculline (1*S*,9*R*) and (−)-bicuculline (1*R*,9*S*) correspond with the known binding affinity of these compounds to GABA receptors, suggesting GABA/glycine sensitive receptor-based feeding deterrence is highly stereoselective (Mullin et al. 1992). Whether stereochemical variation in STL causes differential antagonism of GABA/glycine sensitive receptors is currently unknown, but is an intriguing avenue for future research.

Further study of STL stereochemistry could shed light on macroevolutionary patterns in plant–herbivore interactions. For example, both *cis*- and *trans*-fused STL are widely distributed across the Asteraceae; of the 192 genera sampled, 24 (12.5 %) are only known to produce *cis*-fused STL, 123 (∼64 %) are only known to produce *trans*-fused STL, and 45 (∼23 %) are known to produce both types (unpublished analysis of data in Seaman 1982; Budesínský and Saman 1995). We reason that patterns of STL diversification may be linked to herbivore attack, and make the testable prediction that Asteraceae clades dominated by *cis*- vs. *trans*-fused STL have historically been attacked by different herbivore groups and/or have experienced different levels of attack.

Acknowledgments For invaluable assistance we thank Spencer Behmer, Marion Le Gall, Paul Lenhart, Alexander Zaykov, Jorge Fallas, Ron Parry, Steve Hovick and Evan Siemann. Funding was provided by a Wray-Todd Fellowship, Sigma Xi GIAR, NSF DEB 0716868 and NSF DEB 1011661.

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