PERGAMON

Phytochemistry 58 (2001) 739–742

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

Excretion of miserotoxin and detoxification of the aglycone by grasshoppers (Orthoptera: Acrididae)☆

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Received 19 February 2001; received in revised form 5 June 2001

Abstract

Two species of grasshoppers (*Melanoplus bivittatus* and *M. sanguinipes*) tolerated high levels of miserotoxin (3-nitro-1-propyl-β-D-glucopyranoside) in their diet. Miserotoxin is a causative agent in cattle poisoning when timber milkvetch (*Astragalus miser*) is consumed. Toxic effects were averted by grasshoppers in part by excretion of the intact glycoside. When the aglycone was administered, detoxification was achieved by two routes: by oxidation of the aglycone to 3-nitropropionic acid which was then conjugated with glycine, and by glucosylation of the aglycone to miserotoxin, in each case followed by excretion. Crown Copyright © 2001 Published by Elsevier Science Ltd. All rights reserved.

Keywords: *Astragalus miser*; Fabaceae; Timber milkvetch; Chemical defense; Conjugation; Aliphatic nitro compounds; Amino acids; Melanoplus; Acrididae

1. Introduction

Miserotoxin (3-nitro-1-propyl-β-D-glucopyranoside), a causative agent in cattle poisoning on rangelands in the western USA, was originally isolated from *Astragalus miser* var. *oblongifolius* (Leguminosae) (Stermitz et al., 1969). The glycoside was subsequently isolated from *A. miser* var. *serotinus* (timber milkvetch), which occurs in the Southern Interior of British Columbia, Canada (Majak and Bose, 1974). Other glycosides of 3-nitropropanol (NPOH) were also identified in timber milkvetch (Long et al., 1992). Miserotoxin is rapidly hydrolyzed by microbial enzymes of the rumen, and the aglycone is absorbed and converted to 3-nitropropionic acid (Muir et al., 1984), a potent inhibitor of mitochondrial enzymes essential to respiration (Alston et al., 1977). 3-Nitropropionic acid (NPA) is also implicated as a causative agent of neuronal degeneration in mammals (Alexi et al., 1998).

Previously we reported on the lack of NPA toxicity to melanopline grasshoppers (*Melanoplus bivittatus* and *M. sanguinipes*; Orthoptera: Acrididae), and their metabolic ability to detoxify NPA by way of conjugation with amino acids. The NPA was excreted in the frass (dry fecal pellets) as amides of glycine, L-serine and L-glutamate (Majak et al., 1998). Karakin, a glucose triester of NPA, was also detoxified after hydrolysis in vivo and conjugation of the NPA to the same amino acids (Majak et al., 1998). In feeding trials with the same species of grasshoppers using freeze-dried timber milkvetch, none of the grasshoppers died, nor did they show any sign of altered behavior or disability after subsisting on *A. miser* for 96 h at room temperature (D.L. Johnson, unpublished data). The objectives of this study were to further explore the resistance of grasshoppers to miserotoxin, and to determine the metabolic fate of the aglycone.

2. Results and discussion

2.1. Experiment 1

The concentration of miserotoxin in the timber milkvetch was found to be 4.5–5.2% on a dry-matter basis.

☆ This work was completed by D.L. Johnson et al., for the Department of Agriculture and Agri-Food, Government of Canada © Minister of Public Works and Government Service Canada 2001.

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PII: S0031-9422(01)00310-7

The individual consumption rate of the milkvetch was approximately 40 mg/day, per grasshopper, which is a typical rate of feeding on finely ground dried plant material at room temperature. The rate of intake of miserotoxin was equivalent to approximately 2 mg of the glycoside per grasshopper per day. There was no mortality in treated or untreated groups, and there was no other signs of toxicity.

2.2. Experiment 2

Typically, more than 90% of the food that was treated with the purified glycoside and provided in feeding trays was consumed by the two-striped grasshoppers during the assessment period. The average intake of miserotoxin was estimated at approximately 4.5–4.8 mg per grasshopper per day. There was no mortality or other sign of toxicity. The frass was extracted and miserotoxin was detected on silica gel TLC.

2.3. Experiment 3

No grasshoppers died in the treated groups (a total of one grasshopper in the control groups died), and no other changes in behavior that would indicate possible toxicity were observed. The average amount of the glycoside provided was 11.5 mg per grasshopper, per day, but because of losses (estimated at ca. 20%) in tumble-treatment of the lettuce, and quantities remaining on unconsumed food, the amount of glycoside was estimated to be 6–7 mg per grasshopper. By far, the major component in the frass giving a colour test specific for aliphatic nitro compounds (Majak and Bose, 1974) was miserotoxin, as evidenced by co-chromatography with an authentic standard. The identity of the glycoside as a nitro alkyl excretion product (3.5% of the frass on a dry-matter basis) was confirmed by ^{1}H and ^{13}C NMR spectroscopy. Only a small portion ($<10\%$) of the administered miserotoxin was recovered from the frass, which suggested other pathways of elimination or reductive detoxification (although this feeding experiment was not intended to provide quantitative analysis of fate of all products). Some anaerobic bacteria can reduce the nitro alkyl moiety to the amino group (Anderson et al., 1993), and sequestration of the intact glycoside could also be possible (Duffey, 1980). However, extraction of the grasshopper cadavers and TLC screening did not reveal endogenous accumulation of ninhydrin-positive or nitro alkyl-positive products.

There was no evidence of the aglycone on TLC, suggesting the absence of β-glucosidase activity in *M. bivittatus*, and a possible avoidance of toxicity, through the elimination of the intact glycoside. The low level of β-glucosidase activity agrees with earlier tests that indicated the absence of cyanide formation when homogenates of *M. bivittatus* were incubated with amygdalin (W. Majak, unpublished data). However, a second, minor spot was observed on the TLC that was also visualized with the reagent for nitro compounds. Its R_f was consistent with that of an amino acid conjugate of NPA. This result suggested partial hydrolysis of the glycoside by the insect, or possibly by microflora of the gut, and then oxidation of the aglycone to NPA before conjugation.

2.4. Experiment 4

The average intake of the aglycone was approximately 0.5 mg per grasshopper per day. There was no mortality or other sign of toxicity, and no behaviour indicating disorientation or loss of appetite. When the frass was extracted and fractionated, an amino acid conjugate of NPA was isolated, which was identified by NMR as the glycyl amide of NPA. Clearly, it was demonstrated that *M. bivittatus* had the capacity to oxidize the aglycone, and to detoxify the NPA through conjugation with glycine. There was no evidence of excess NPOH in the frass.

However, the more common method of NPOH detoxification by *M. bivittatus* appeared to be through glucosylation to form the β-D-glucoside, which, in effect, is miserotoxin. Based on colour intensity on TLC, the ratio of the glycoside to the amino acid conjugate appeared to be approximately 2:1. On a molar basis, the yield of the glycoside (25 mg) accounted for approximately 10% of the administered aglycone. This type of glucosylation reaction has long been recognized in higher plants. When simple phenols, such as phloroglucinol or phenol itself, are administered to higher plants, they are detoxified by conversion to the corresponding glucosides (Towers, 1964). Thus, there are now three methods for the metabolic detoxification of the miserotoxin aglycone: (1) conjugation after oxidation; (2) glucosylation and (3) reduction of the nitro group to the much less toxic amino group which is effected by rumen bacteria (Anderson et al., 1993).

3. Experimental

3.1. General experimental procedures

NMR spectra were measured with a Bruker AM-400 spectrometer at 400 MHZ (^{1}H) and 100 MHZ (^{13}C) of samples dissolved in D_2O or MeOH-d4. The TLC solvent system on silica gel was chloroform–methanol (3:1) with 0.2% HCOOH and the R_f's were 0.32 for miserotoxin and 0.30 for the glycyl conjugate. The ^{1}H and ^{13}C NMR spectra of these compounds were as reported (Majak et al., 1998; Benn and Majak, 1989).

3.2. Biological materials

The grasshoppers used were the first captive generation produced from eggs laid by field-collected adult grasshoppers. [The grasshoppers we used were not from

the widely used non-diapause *Melanoplus sanguinipes* laboratory strain (Pickford and Randell, 1969), which have been shown to have enhanced detoxicative enzyme activity: Isman et al., 1996]. Grasshoppers used in toxicity assessments and feeding trials were confined in cages constructed of screen, glass and wood and supplied with adequate air flow and lighting (photoperiod 15 h light, 9 h dark; measured temp. 27–29 °C day, 22–24 °C night). Methods of rearing and handling of grasshoppers, and of assessment of grasshopper feeding, were similar to those described by Johnson and Pavlikova (1986) and Lactin and Johnson (1995). *A. miser* var. *serotinus* specimens were field collected in the Southern Interior of British Columbia. Specimens were freeze dried and ground prior to grasshopper feeding.

3.3. Experiments

3.3.1. Experiment 1: consumption and gross toxicity

Groups of 12 grasshoppers (repeated separately for *M. bivittatus* and *M. sanguinipes*, two complete replications for each species) were each confined with 1 g of freeze-dried *A. miser* var. *serotinus*, which they consumed within 48 h. Feeding behavior and frequency was observed in order to confirm that all individuals were consuming the plant material. At the end of 48 h, a second gram of freeze-dried *A. miser* var. *serotinus* was added to each cage and again it was consumed within 48 h. The concentration of miserotoxin in the timber milkvetch was determined by HPLC (Quinton et al., 1989). The experiment was subsequently repeated in four complete replications to confirm results regarding gross toxicity.

3.3.2. Experiment 2: intake and toxicity of the purified glycoside

A total of 40 fifth-instar *M. bivittatus* were confined in cages with wheat bran that was treated with (or without) purified miserotoxin, which was dissolved in water. (Treatment identifiers were coded, and unknown to handlers and observers during the assessments.) The grasshoppers were confined with measured quantities of the wheat bran provided in feeding trays each day for 8 days. The administered miserotoxin was isolated from timber milkvetch by conventional procedures (Majak and Bose, 1974; Majak et al., 1992), but continuous ethyl acetate extraction was used before column chromatography. To detect the glycoside in the frass, 1 g of it was extracted with hot ethanol and the concentrate was fractionated on silica gel column chromatography (25×2 cm) and centrifugally accelerated radial TLC (Chromatotron) as described previously (Majak et al., 1998).

3.3.3. Experiment 3: assessment of intake, excretion and fate of the glycoside

In the third experiment, the purified glycoside was applied to a mixture of lettuce leaves (streaking with pipette), and to wheat bran (stirred and mixed by tumbling until evenly absorbed). A total of 80 two-striped grasshopers (*M. bivittatus*) were confined with the treated feed in cages for 4 days. The grasshoppers were kept alive for an additional 7 days after that time, to observe any possible delayed mortality. The collected frass was extracted and fractionated by conventional procedures (Majak et al., 1998). Individual fractions were screened by TLC.

3.3.4. Experiment 4: intake of the aglycone and fate of NPOH

In a fourth experiment, the aglycone was fed to grasshoppers to determine the metabolic fate of NPOH. A total of 50 two-striped grasshoppers (*M. bivittatus*) were confined in feeding cages and fed aglycone for 6 days. 3-Nitropropanol was synthesized from 3-bromopropanol by Gary Yost, University of Utah. Frass from the grasshoppers (0.93 g) was extracted as described above.

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