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DETOXIFICATION OF 3-NITROPROPIONIC ACID AND KARAKIN BY MELANOPLINE GRASSHOPPERS§

IN HONOUR OF PROFESSOR G. H. NEIL TOWERS 75TH BIRTHDAY

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Abstract—Two species of melanopline grasshoppers (*Melanoplus bivittatus* and *M. sanguinipes*) tolerated high levels of 3-nitropriopionic acid (NPA) in their diet (LD_{30} = 3 μg g^{-1}). The NPA was absorbed, conjugated to glycine, L-serine or L-glutamate and excreted in the frass as amides. Karakin was also consumed without ill effects. Karakin, a glucose triester of NPA, was apparently hydrolyzed *in vivo*, and the NPA was conjugated to the same amino acids. The conjugates were identified by NMR and ORD spectroscopy.

INTRODUCTION

The occurrence of 3-nitropropionic acid (NPA) in higher plants has been frequently reported [1–4], especially in the species of *Astragalus* (Leguminosae) but it is generally accepted that the major pool of NPA is bound, esterified to glucose as mono-, di-, tri- or tetra-congeners of NPA [5, 6]. In 1872, Skey [7] reported the isolation of karakin from untreated karaka fruit (*Corynocarpus laevigata*), a staple food of the Maori, which was carefully baked and washed before consumption. This was the first isolation of a natural product containing the nitro function but its structure was only elucidated a century later and it proved to be 1,2,6-tris-*O*-(3-nitropropanoyl)-β-D-glucose [8]. Karakin is often the major product when esters of NPA are isolated [9, 10]. Karakin is rapidly hydrolyzed in mammalian tissues releasing NPA, a potent inhibitor of mitochondrial enzymes essential to respiration in mammals [11]. NPA is also toxic to a number of insects [11] including the domestic honey bee [12]. However in feeding trials with melanopline grasshoppers using freeze-dried *Astragalus collinus*, which contains high levels of karakin, none of the grasshoppers died, nor did they show any sign of disability after subsisting on *A. collinus* for 96 h at room temperature (Johnson, unpublished data). The objective of this study was to demonstrate the resistance of grasshoppers to NPA, to elucidate the metabolic fate of NPA in two species of melanopline grasshoppers and to propose a biochemical pathway for the detoxification of karakin.

RESULTS AND DISCUSSION

When NPA was administered at 4.0 to 80 μg/g, the mortality rate was <2%, which is well within natural control mortality for *Melanoplus bivittatus* (Table 1). Lettuce wafers coated with NPA were all consumed at rates of up to and including 800 μg NPA/g. The consumption was slightly delayed at 2400 μg/g and there were two rejections (Table 1). There was a 20% rejection rate and a 33% mortality rate at 3200 μg/g. It was not possible to determine an accurate LD_{50} because the grasshoppers could not consume enough NPA in a short time. A computer-generated LD_{50} suggested it would be about 16,700 μg/g. An estimate of an LD_{30} was within the bounds of the data and at that level of mortality the dose was 3095 μg/g (95% fiducial limits: 1822 to 7300 μg/g). In rats, the LD_{50} for NPA is 67 μg/g [13].

Migratory grasshoppers (*M. sanguinipes*) and two striped grasshoppers (*M. bivittatus*) were fed freeze-dried *Astragalus collinus* powder for 4 days. The con-

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§ Dedicated to Professor Neil Towers on the occasion of his seventy-fifth birthday.

Table 1. Number of mortalities in *Melanoplus bivittatus* fed NPA at 0 to 3200 μg/g

Dose* μg/g	N	Number dead	% dead
0	60	0	0
0.4	60	0	0
4.0	60	1	1.67
40	60	1	1.67
80	60	1	1.67
400	60	10	16.67
600	60	11	18.33
800	59§	10	16.95
1600	60	9	15.00
2400	58†	17	29.31
3200	48†	16	33.33

* Doses of NPA were applied on lettuce wafers and grasshoppers were fed individually.

§ One died following some moulting damage related to contact with the container during the feeding and assessment.

† Smaller groups due to rejection of diet.

centration of karakin was 0.3%. The individual consumption rate was approximately 40 mg d^{-1} which is a typical rate of feeding on plant powder at room temperature. Extraction of the cadavers with hot ethanol followed by TLC failed to reveal the presence of aliphatic nitro compounds. Similarly, NPA or metabolites of NPA were not detected in extracts of carcasses of *M. bivittatus* fed NPA at 800 μg/g body weight. However, compounds giving colour tests specific for nitro compounds [14] were visualized on TLC when ethanolic extracts were prepared from the frass of *M. bivittatus*. Large scale feeding trials were then conducted with *M. bivittatus* and *M. sanguinipes* and the frass was collected from groups of 100 grasshoppers.

Chromatographic separations of the frass on silica gel yielded three amino acid conjugate of NPA, namely the glycyl, L-seryl and L-glutamyl amides of NPA. The glycyl conjugate was a major component of the frass occurring as ca 1% of the dry weight whereas the other two were present at <0.5%. The pathway of NPA detoxification in the grasshoppers thus resembles that of aromatic carboxylic acids in other insects, which excrete them as their amidic conjugates with glycine and other α-amino acids [15, 16]. NPA is an isoelectric analog of the Krebs cycle intermediate succinate and it is an inhibitor of aerobic cellular respiration causing irreversible blockage of the cycle [17, 18]. Amide formation at the carboxyl function of NPA may well impair the toxic mode of action of NPA since the structural analogy to succinate would be seriously disrupted. Another mechanism for NPA detoxification has also been reported in rumen bacteria whereby the nitro function is reduced under anaerobic conditions to form β-alanine [19] but this product was not detected in the present study.

The NPA conjugates were initially isolated from the frass of *M. sanguinipes* but ethanolic extracts of the frass of *M. bivittatus* fed NPA also yielded the same conjugates as shown by cochromatography on TLC. The NPA conjugates were also detected in frass by TLC when karakin was administered to grasshoppers indicating the presence of esterase activity. Whether this activity was associated with the gut microflora of grasshoppers, or whether it is localized in tissues remains to be seen. General esterase activity has been detected in gut tissues of *Melanoplus* spp. [20]. So, detoxification of karakin and probably other glucose esters of NPA by melanopline grasshoppers most probably involves hydrolysis of the esters and the immediate conjugation of the free NPA with amino acids.

EXPERIMENTAL

NMR spectra were measured with a Bruker AM-400 spectrometer at 400 MHz (^1H) and 100 MHz (^{13}C). ORD spectra were recorded with a JASCO model 715 spectropolarimeter.

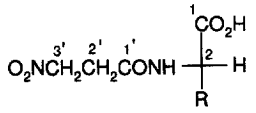
LD_{50} studies with grasshoppers

Grasshoppers were hatched from eggs laid in the laboratory by adult *M. bivittatus* and *M. sanguinipes* collected in pastures and roadsides near Iron Springs, Alberta in August 1996. The methods involved in the oviposition, storage, termination of diapause, hatching and handling were as described previously [21]. Groups of 60 individually caged third instar two-striped grasshoppers (*M. bivittatus*) maintained at 27° were fed NPA in 10 increments from 0 to 3200 μg g^{-1} body weight (Table 1). Each dose of NPA was applied as a 4 μl droplet in water to a 0.8 cm diameter wafer of iceberg lettuce and the droplet was allowed to dry before feeding the grasshoppers individually. Mortality was assessed daily for 4 days. the LD_{30} and LD_{50} were estimated by probit analysis, which gave a good fit to the data (Pearson Goodness-of-Fit Chi-square, $P > 0.45$).

Isolation of NPA conjugates from frass

After feeding NPA. After a 4-day feeding period, 3 g of frass was collected from 100 migratory adult grasshoppers fed NPA in a mixture of bran and lettuce. The daily intake of NPA was 2 to 3 mg per grasshopper. The frass was freeze-dried and extracted with hot ethanol. The extract was filtered, concentrated to dryness and the residue (214 mg) was redissolved in a small volume of methanol. This was applied to a silica gel column (3 × 20 cm) equilibrated in $CHCl_3$. The column was developed with $CHCl_3$–MeOH (4:1 → 1:4, 8 × 50 ml) and then MeOH (7 × 50 ml). Individual fractions were concentrated to dryness and assayed for aliphatic nitro compounds by TLC on silica gel ($CHCl_3$–MeOH–HCOOH, 50:50:1) using

the diazotized *p*-nitroaniline spray reagent for detection purposes [14]. Fractions containing the glycine-NPA conjugate (**1**) ($R_f = 0.64$) were combined and purified by centrifugally accelerated radial TLC (Chromatotron), on a 1 mm silica gel 60 coated rotor, using a stepwise gradient of $CHCl_3$–MeOH (9:1 → 1:1) containing 0.2% HCOOH and flow rate of 2.5 ml min^{-1}. Fractions from the column containing the serine-NPA conjugate (**2**) ($R_f = 0.56$) were purified on the Chromatotron using a stepwise gradient of $CHCl_3$–MeOH (9:1 → 6:4) with 0.2% HCOOH. This fractionation also yielded a mixture of the serine-NPA and glutamate-NPA ($R_f = 0.43$) conjugates and the latter (**3**) was purified by a second development in the same solvent.



1 R=H

2 R=CH_2OH

3 R=$CH_2CH_2CO_2H$

NMR data

For **1** (in D_2O with MeOH as internal Ref. δ_H 3.31, δ_C 49.1) 4.84 (2H, *t*, $J = 6$ Hz, H-3′), 3.83 (2H, *s*, H-2), and 3.08 (2H, *t*, $J = 6$ Hz, H-2′); δ_C 176.9 *s*, 171.9 *s*, 70.7 *t* (C-2′), 43.6 *t* (C-2) and 32.1 *t* (H-2′).

For **2** (in MeOH-d_4 plus a drop of ca 5% CF_3CO_2H in D_2O) δ_H 4.73 (2H, *br t*, $J = 6.5$ Hz, H-3′), 4.51 (1H, *br t*, J ca 4.5 Hz, H-2), 3.92 (1H, *dd*, $J = 4.9$ and 11.3 Hz, H-3A), 3.84 (1H, *dd*, $J = 4.0$ and 11.3 Hz, H-3B), 3.01 (2H, *m*, H-2′). Selective decoupling experiments established the individual proton resonances. Thus irradiation at δ_H 4.51 reduced both of the resonances at δ_H 3.92 and 3.84 to doublets ($J = 11.4$ Hz). Similarly irradiation at δ_H 4.7 collapsed the multiplet at δ_H 3.01 to a broad singlet, while conversely irradiation at δ_H 3.01 collapsed the triplet at δ_H 4.7 to a broad singlet.

For **3** (in MeOH-d_4 plus a drop of ca 5% CF_3CO_2H in D_2O) δ_H 4.73 (2H, *t*, $J = 6.7$ Hz, H-3′), 4.42 (1H, *dd*, $J = 4.6$ and 9.2 Hz, H-2), ca 2.97 (2H, *m*, H-2′), 2.34 (2H, *t*, $J = 7.6$ H, H-4), 2.2 (1H, *m*, H-3A) and 2.0 (1H, *m*, H-3B). COSY and selective decoupling experiments established the individual assignments.

After feeding karakin. Karakin, obtained in a previous study [9], was combined with wheat bran (sieved to provide a flake size range of 1–3 mm diam., ca 9% moisture) and tumbled for 2 h. The resulting bait was readily consumed by two groups of 20 grasshoppers. The daily rate of consumption was 2 to 3 mg of karakin per grasshopper (*M. sanguinipes*). The frass was collected after 6 days of feeding. It was extracted and fractionated as above, and TLC revealed the presence of the same three conjugates.

Hydrolysis of the conjugates, and formation of DNP derivatives

The glycyl, seryl, and glutamyl conjugates of NPA were hydrolyzed in 6N HCl (20 h, 85°) and the amino acid residues were derivatized with 1-fluoro-2,4-dinitrobenzene [22]. The dinitrophenyl (DNP) derivatives of the hydrolyzed conjugates cochromatographed on silica gel with DNP-glycine, DNP-L-serine and DNP-L-glutamate prepared from amino acid standards [23]. Comparison of the ORD spectra of the conjugate-derived DNP-serine and DNP-glutamic acid with the literature [24], and those of the standards, revealed that they were largely, if not entirely, unracemized L-compounds.

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