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# CHROMATOGRAPHIC METHODS FOR THE ISOLATION OF MISEROTOXIN AND DETECTION OF ALIPHATIC NITRO COMPOUNDS

WALTER MAJAK
Research Station, Canada Agriculture, Kamloops, British Columbia, Canada*

and

R. J. BOSE
Fisheries Research Board of Canada, Vancouver 8, British Columbia, Canada

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**Key Word Index**—*Astragalus miser*; Leguminosae; timber milkvetch; miserotoxin; aliphatic nitro compounds; purification and detection methods.

**Abstract**—A facile chromatographic procedure is described for the isolation and purification of miserotoxin. New spray reagents are demonstrated for aliphatic nitro compounds and a colorimetric method is developed which is sensitive to 1 ppm miserotoxin.

## INTRODUCTION

LIVESTOCK poisoning resulting from the ingestion of *Astragalus miser* var. *serotinus* (timber milkvetch) is attributed to the natural occurrence of miserotoxin (3-nitro-1-propyl-β-D-glucopyranoside[1–4]). Stermitz *et al.*[1] conducted the original isolation and characterization of the toxin. Their purification procedure involved the use of automatic countercurrent distribution and the method proved to be most difficult due to the presence of interfering flavonoids.[5] Hahn isolated the aglycone of miserotoxin using Sephadex G10.[6] The yield was extremely low (0·03% dry wt) however, and the author indicated the possibility of enzymatic hydrolysis of the glucoside during the aqueous extraction.[6] A simpler, more sensitive method was required for isolation and purification of miserotoxin.

Chick, rat and mouse bioassays have been used to detect toxic fractions of *A. miser*[7–9] and *Indigofera endecaphylla*[10,11] and the latter was found to contain 3-nitropropanoic

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* Contribution No. 241.

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acid,[10] ethyl-3-nitropropanoate and a series of nitropropanoate esters of glucose, the endecaphyllins.[12-14] Recently, Stermitz *et al.* isolated 1,6-di-3-nitropropanoyl-β-D-glucopyranoside (cibarian[15]) from *Astragalus cibarius.*[16] Matsuo *et al.* have reported the natural occurrence, using GLC, of 1-nitro-2-phenylethane as a possible intermediate of benzylglucosinolate biosynthesis in *Tropaeolum majus.*[17] Aliphatic nitro compounds are usually detected by various adaptations of the classical Griess–Ilosvay test for nitrous acid:[18] a preliminary alkali treatment of the compound (or extract) yields a small quantity of nitrite ion which is spectrophotometrically measured after diazotization of sulfanilic acid and coupling with 1-naphthylamine. Since the procedures based on the release and measurement of the nitrite ion[7,10,19,20] require an extended alkaline treatment, these methods are not suitable as sensitive and specific chromatographic spray reagents.

The present work describes a simplified chromatographic procedure for the isolation of miserotoxin in high yield, new specific spray reagents for aliphatic nitro compounds and a direct colorimetric method which is sensitive to 1 ppm miserotoxin.

## RESULTS

### *Isolation and identification of miserotoxin*

As described in the Experimental, miserotoxin was isolated on a large scale by purifying the crude extract with various adsorbents which bind phenolic constituents followed by PC and TLC of the clarified eluates. We obtained miserotoxin as a light yellow syrup in 0·33% (fr. wt) yield from *A. miser* var. *serotinus*. Stermitz *et al.*[4] reported a 2·6% (dry ground material) yield from *A. miser* var. *oblongifolius*.

Acid hydrolysis[4] of the isolated compound yielded glucose and 3-nitropropanol. Glucose was identified by its color reactions with *p*-anisidine phthalate and aniline diphenylamine phosphate and by co-chromatography with a standard. The aglycone, 3-nitropropanol, was identified by its color reactions with diazotized *p*-nitroaniline and diazotized sulfanilic acid and by co-chromatography with authentic 3-nitropropanol. The IR spectrum of the isolated compound (1–2 mg/100 mg KBr) was in complete agreement with that reported by others for miserotoxin.[4,21] The MS spectrum of the trimethylsilyl derivative agreed with the assigned structure for the isolate. The highest mass fragment ion appeared at *m/e* 540 corresponding to M-15 characteristic of the MS of trimethylsilyl sugar derivatives.[22] Other intense fragment ions, typical of monosaccharide trimethylsilyl ethers[22] appear in the spectrum: *m/e* 217, 204 (base peak), 191, 147 and 73. The fragment ion at *m/e* 88 had a relative abundance of 20% and corresponded to the fragment ion $(CH_2CH_2CH_2NO_2)^+$ generated from the aglycone.

The 100 MHz NMR spectrum of the isolated compound (in $D_2O$/DCl) showed a multiplet at 2·66–2·90 δ, a triplet at 5·12 δ (*J* 6 Hz) and a doublet at 4·90 δ (*J* 7·5 Hz)

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from external TMS. The corresponding $\delta$ values as reported by others[1] ($q$ 2·34 $\delta$, $t$ 4·68 $\delta$ and $d$ 4·51 $\delta$: 60 MHz NMR) are discrepant. The 100 MHz spectrum (in $CDCl_3$) of the trimethylsilyl derivative of the isolate however, was well-resolved: 2·12 $\delta$ (q, 2H, $J$ 6 Hz, $-CH_2C\underline{H}_2CH_2NO_2$); 3·97 $\delta$ ($d$, 1H, $J$ 7·5 Hz, anomeric $\alpha$ proton of a pyranose sugar) and 4·29 $\delta$ ($t$, 2H, $J$ 6·5 Hz, $-C\underline{H}_2NO_2$) and these values are in good agreement with those reported by Norris.[21]

### *Chromatography and color reactions of aliphatic nitro compounds*

The chromatographic spray reagents employing diazotized $p$-nitroaniline (system A) and diazotized sulfanilic acid (system B) are based on the reaction in which benzenediazonium salts couple with nitroethane in alkaline solution.[23] This reaction is limited to primary and secondary aliphatic nitro compounds which possess an $\alpha$ hydrogen(s). When system A was applied, the initial reactions on miserotoxin and 3-nitropropanol yielded a burgundy-red color whereas 3-nitropropanoic acid, 1-nitro-2-phenylethane and cibarian produced an orange–red coloration (on Avicel TLC plates). System B, on the other hand, resulted in the initial formation of orange–yellow spots following treatment of the five naturally-occurring aliphatic nitro compounds listed in Table 1. If the final conditions in systems A and B are acidic, stable yellow colors are produced. Alternately, alkaline conditions produce unstable shades of red (see above) which change to green on standing (system A) and orange–yellow spots (system B).

TABLE 1. CHROMATOGRAPHIC PROPERTIES OF ALIPHATIC NITRO COMPOUNDS

| | $R_f \times 100$ | | | | | |
|---|---|---|---|---|---|---|
| | Solvents* | | | | | |
| Compound | BAW | EPW | BEW† | Pyr | BzAW† | PrOH |
| Miserotoxin | 36 | 45 | 55 | 60 | 3 | 66 |
| 3-Nitropropanol[a] | 81 | 86 | 89 | 82 | 65 | 83 |
| 3-Nitropropanoic acid | 77 | 31 | 87 | 29 | 55 | 53 |
| 1-Nitro-2-phenylethane[b] | 93 | 95 | 100 | 91 | 100 | 92 |
| Cibarian[c] | 42 | 77 | 63 | 73 | 3 | 75 |

* *Key.* BAW, $n$-BuOH–HoAc–$H_2O$ (4 : 1 : 5, upper phase); EPW, EtOAc–pyridine–$H_2O$ (8 : 2 : 1); BEW, $n$-BuOH–EtOH–$H_2O$ (4 : 1 : 5, upper phase); Pyr, $n$-BuOH–pyridine–$C_6H_6$–$H_2O$ (5 : 3 : 1 : 3); BzAW, $C_6H_6$–HoAc–$H_2O$ (10 : 7 : 3); PrOH, $n$-PrOH–$H_2O$ (7 : 3). $R_f$s were determined on Whatman No. 3 MM chromatography paper unless otherwise indicated.

† On Avicel.

[a] Courtesy of Dr. W. C. Williams; [b] Courtesy of Dr. E. W. Underhill; [c] Courtesy of Dr. F. R. Stermitz.

The spray system using $FeCl_3$ under acidic conditions (system C) is a modification of Scott and Treon's colorimetric method[24] which is based on the colored complex formed between the *aci* form of primary aliphatic nitro compounds and the ferric ion.[25] When system C was applied the aliphatic nitro compounds (listed in Table 1) exhibited a transient orange color which developed gradually to purple–gray on a bright yellow background.

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Miserotoxin could be readily detected in applications of 3 μg/cm$^2$ (system A), 10 μg/cm$^2$ (system B) and 40 μg/cm$^2$ (system C) on 0·5 mm Avicel TLC plates. Table 1 gives the $R_f$ values of five aliphatic nitro compounds in various solvents.

*Quantitative determination of miserotoxin*

Using Williams and Norris' modification[7] of Cooke's method[10] we found that miserotoxin could be detected at a concentration of 50 μg/ml. Matsumoto *et al.*[19] enhanced the sensitivity of the classical procedures by condensing formaldehyde with 3-nitropropanoic acid and this was followed by a postulated β-elimination of the nitro group[20] (pH 9·5, 15 p.s.i.). Although the latter method[19] is sensitive to 1 μg/ml 3-nitropropanoic acid, we have found that at least 20 μg/ml 2-nitropropanol or 20 μg/ml miserotoxin are required to give a significant colorimetric reading. Furthermore, when the above methods are applied to crude extracts, the nitrite ion which is measured reflects the total aliphatic nitro concentration. We have observed also, that when Cooke's method[7,10] is employed with crude extracts of *A. miser* var. *serotinus* the expected red azo dye (formation of α-naphthylamine-*p*-azobenzene-*p*-sulfonic acid[26]) is not obtained but rather a salmon pink color ($\lambda_{max}$ 525 nm). If the crude extract is partially purified (polyamide column) however, and then tested for aliphatic nitro compounds[7,10] the cherry red derivative is obtained at a significantly higher concentration. In addition we have found that a red azo dye ($\lambda_{max}$ 525 nm) can be formed directly (i.e. without prior alkaline treatment for $NO_2^-$ production) by reacting the Griess–Ilosvay reagent with primary aliphatic nitro compounds such as miserotoxin and 3-nitropropanoic acid (*ca* 500 μg/ml). Accordingly, the various complicating features described above initiated the development of a new quantitative method.

TABLE 2. MISEROTOXIN CONTENT OF *Astragalus miser* VAR. *serotinus*

| Collection date 1972 | % Miserotoxin* | |
|---|---|---|
| | Wet site | Dry site |
| May 26 | 3·5 | 4·1 |
| June 5 | 3·2 | 4·4 |
| June 12 | 3·3 | 3·5† |
| June 20 | 3·1† | 3·0‡ |
| June 28 | 2·8† | 2·8§ |

* Based on dry, ground material.
† Full flower stage.
‡ Beyond full flower.
§ Seed pod stage.

Our procedure permits a quick TLC micro-isolation of miserotoxin from crude extracts. The specific band is eluted and measured by coupling to diazotized *p*-nitroaniline. When a standard miserotoxin solution is treated according to our procedures for extraction and TLC the recovery is 100%. The absorbance maximum (405 nm) of the derivatized miserotoxin is stable for 30 min (a 5% reduction in absorbance is observed after 90 min). The stability of the derivative can be affected however, by direct illumination from an intense light source and conditions of diffuse, fluorescent light are recommended during

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[26] VOGEL, A. I. (1943) *Quantitative Inorganic Analysis*, p. 700. Longmans, Green, London.

the derivatization. This procedure is sensitive to 1 $\mu$g/ml miserotoxin and 0·5 $\mu$g/ml 3-nitropropionic acid.

Table 2 describes the values which we obtained for miserotoxin concentrations of *A. miser* var. *serotinus* collected near Kamloops, B.C. during the summer of 1972. The results indicate that miserotoxin levels in *A. miser* var. *serotinus* are highest just prior to the full flower stage. Similarly, Williams and Norris[7] (using Cooke's method[10]) previously reported values of 3·2% (prebloom stage) and 2·7% (bloom stage) for the same variety of timber milkvetch. The authors reported, however, that *A. miser* var. *oblongifolius* reached its highest miserotoxin levels when the plants were in bloom and past bloom stages of growth and that the levels of the toxin dropped significantly as the pods ripened.[7]

Heretofore, the miserotoxin content of timber milkvetch has been reported from extracts of oven-dried (60°, 48 hr) and ground plant samples[2,7,27,28] (see also Table 2). It is noteworthy to mention that in recent experiments we have found a 12% (±2%) increase in miserotoxin content determined from the extraction of fresh, frozen material (as compared to a parallel extraction of oven-dried material).

## EXPERIMENTAL

*Plant material.* Shoots of *A. miser* var. *serotinus* were collected near Kamloops, B.C. during the summer of 1972. The fresh material was immediately frozen and stored in polyethylene bags at −10°. Oven-dried samples were prepared as described by others.[7]

*Large scale isolation of miserotoxin.* The fresh plant material (1·36 kg) was thoroughly extracted with hot 80% EtOH. The extract was concentrated under vacuum, suspended with Celite in 1 l. hot $H_2O$ and filtered. The filtrate was passed through a column (5·5 × 33 cm) of polyamide (MN Polyamide CC6, equilibrated in $H_2O$), eluted with 1 l, $H_2O$ and the brown eluate was combined with 250 g decolorizing carbon (Norit "A"). The suspension was filtered and the filtrate (turns amber upon the addition of a few drops of AcOH) was concentrated. The concentrate was combined with 3 vols of acetone, the clear supernatant was decanted after 48 hr and concentrated again to remove the acetone. The concentrate was applied to a DEAE-cellulose column ($OAc^-$, 6 × 6 cm, equilibrated in $H_2O$), eluted with $H_2O$ and the eluate was chromatographed successively in the following solvents on Whatman No. 3 MM chromatography paper: BAW, EPW and PrOH. The final chromatographic step proceeded in BEW on 1 mm Avicel plates (20 × 40 cm). At high concentrations miserotoxin can be visualized with a short wavelength UV lamp.

*Spray systems. System A.* Diazotized *p*-nitroaniline was prepared by combining 0·30 ml 5% $NaNO_2$ (in $H_2O$) with 10·0 ml 0·3% *p*-nitroaniline (in N HCl) immediately before use. These proportions minimize the formation of excess $HNO_2$ which can interfere with the quantitative assay. The chromatogram was sprayed lightly with 2 M NaOH–EtOH (1 : 1) and this was followed by an application of diazotized *p*-nitroaniline. The red coloration was produced by over-spraying with alkali and this was converted to yellow with acid. *System B.* 5 ml 5% $NaNO_2$ were combined with 5 ml 0·9% sulfanilic acid (in N HCl) immediately before use. The chromatogram was sprayed lightly with 2 M NaOH–EtOH (1 : 1) and this was followed by diazotized sulfanilic acid. An alkaline overspray produced the orange–yellow coloration which changed to yellow in the presence of acid. *System C.* The chromatogram (free of acidic solvents) was sprayed with 2 M NaOH-EtOH (1 : 1) and dried under hot air for 1 min. The following sprays were applied subsequently, lightly and in rapid succession: (a) 0·1 N HCl, (b) 10% $FeCl_3 . 6H_2O$ (in $H_2O$) and (c) 6 N HCl. The compounds appeared as orange spots changing gradually to purple–gray on a bright yellow background.

*Micro-isolation and quantitative determination of miserotoxin.* Avicel microcrystalline cellulose (TG-101) was washed initially with hot 80% EtOH and acetone. MN-Polyamide-11 was washed with 20% EtOH (in $H_2O$) and MeOH on a coarse sintered glass funnel to remove the fines. A dried and ground plant sample (1·00 g) was extracted 2 × with 100 ml hot 80% EtOH in a Waring blender, filtered and the residue was washed with an additional 100 ml 80% EtOH. The extract was concentrated to dryness, resuspended in a minimum amount of hot $H_2O$, filtered through a bed of Polyamide (Woelm): Celite (1 : 3) and the filtrate was adjusted to 50·0 ml (extract 1). Stratified TLC plates were prepared as follows: A 0·7 × 20 cm channel was cleared 3 cm from one edge (hereupon designated as the lower edge) of a 0·5 mm Avicel plate (20 × 20 cm). 1 ml of an homogenized slurry of MN-Polyamide-11: Avicel (1 g: 1 g in 7 ml MeOH) was poured into the open channel to form a continuous polyamide interlayer. Extract 1, 0·05 ml, was applied as a band between the lower

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[27] Williams, M. C. (1970) *J. Range Manage.* **23**, 400.

[28] Mosher, G. A., Kristnamurti, C. R. and Kitts, W. D. (1971) *Can. J. Anim. Sci.* **51**, 465.

edge and the polyamide interlayer. The plate was developed twice in 20% EtOH (in $H_2O$) until the front migrated 2 cm into the upper Avicel layer. The polyamide interlayer and the cellulose layer beneath were completely removed and development proceeded in BEW. The miserotoxin band, located by the use of adjacent standards, was eluted with *ca* 7 ml $H_2O$ into a 10·0 ml volumetric flask. NaOH (1 M, 0·65 ml) was added, the contents were mixed and combined with 1·0 ml diazotized *p*-nitroaniline and the vol. was adjusted to 10·0 ml. The absorbance was recorded at 405 nm (using a reagent and a TLC blank) and the concentration was determined from a standard curve for miserotoxin (1–10 μg/ml). Since miserotoxin is isolated as a syrup, standard solutions were prepared from miserotoxin which had been dried to a constant weight under high vacuum. The moisture content of the dried miserotoxin was negligible as evidenced by the elemental analysis of the isolate which was consistent with the assigned structure (Found: C, 40·05; H, 6·43; N, 5·17. Calc. for $C_9H_{17}NO_8$: C, 40·30; H, 6·80; N, 5·21%).

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