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ISOLATION OF NITRO COMPOUNDS FROM *ASTRAGALUS* SPECIES

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Key Word Index—*Astragalus canadensis*; *A. flexuosus*; *Leguminosae*; *nitro compounds*; modified Griess-Ilosvay TLC detection method; high pressure liquid chromatography; karakin; miserotoxin.

Abstract—From *Astragalus canadensis* var. *Mortonii* and *A. flexuosus* var. *flexuosus*, two nitropropanoic acid glucosides were isolated: karakin and 1,6-di-(3-nitropropanoyl)-1-β-D-glucopyranose. *A. flexuosus* was also found to contain other nitro compounds including miserotoxin. Two new chromatographic methods were introduced for rapid identification of nitro-containing compounds contained in crude plant extracts. The first involves modification of the Griess–Ilosvay method to produce a spray reagent for visualization of nitro-containing compounds on TLC. The second uses high pressure liquid chromatography with UV detection for nitro-containing compounds separated on a micro-Porasil column.

INTRODUCTION

In continuing our study [1,2] of the nitro compounds of the genus *Astragalus* (Leguminosae), we encountered two species, *A. canadensis* var. *Mortonii* (Nutt.) Wats. and *A. flexuosus* var. *flexuosus* (Hook) Don which showed strong positive tests for aliphatic nitro compounds. We report here their chemical analysis. As the work was progressing, it became evident that good TLC and high pressure liquid chromatographic (HPLC) analyses would assist in this and projected studies, and hence we also investigated the development of such methods.

RESULTS AND DISCUSSION

Dried ground plant material from *A. canadensis* and *A. flexuosus* was extracted with acetone. Concentration of the extract yielded a thick green tar which was extracted with hexane to yield a brown crude plant extract residue. The residues from *A. canadensis* and *A. flexuosus* was chromatographed on silica gel columns eluted with acetone in chloroform (0–80%). Fractions were collected, dried and NMR spectra were determined and each was tested for nitro-containing compounds by the Griess–Ilosvay method.

In both species, two well separated nitro-containing compounds were found in especially large centrations. The first, found in early fractions, was shown to be 1,6-di-(3-nitropropanoyl)-1-β-D-glucopyranoside (cibarian) previously isolated [2] from *A. cibarius*. The second compound (in the middle fractions) was shown to be 1,2,6-tri-(3-nitropropanoyl)-l-β-D-glucopyranoside (karakin) m.p. 124–125. From the MS (methane) the MW was found to be 484 and it could be deduced that the compound was a triester of 3-nitropropanoic acid with glucose. The NMR showed that the anomeric C-l position was β-substituted on the glucose ring and that the 6-position was esterified as well. Samples of karakin isolated by Carter [3] and by Finnegan and Mueller (endecaphyllin B) [4] were found to be identical to our unknown which is thus the 1,2,6-triester [4–6]. We independently investigated karakin structure by periodate oxidation and found the same results as Finnegan and Stephan [5,6], considerable over-oxidation being observed. This makes the method somewhat less than perfect for structure work.

Table 1. R_f Values for TLC separation of nitro compounds from *A. flexuosus*

Compound	Relative amount present	R_f Value (1:1 $CHCl_3$–acetone)	R_f Value (5:3 Benzene–MeOH)
Miserotoxin	+ +	0·04	0·32
Cibarian	+ +	0·09	0·36
Karakin	+ + + +	0·23	0·53
Hiptagin	+	0·34	0·39
3-Nitropropanol	+	0·46	0·25
3-Nitropropanoic acid	+ + +	0·63	0·58

Table 2. Retention times* for HPLC separation of nitro compounds from *A. flexuosus*

Compound	Amount present† % dry wt	Retention time (min) 7:3 Et_2O–acetonitrile	Retention time (min) 5:5:3 Hexane–Et_2O–acetonitrile
Miserotoxin	0·24	1·86	4·52
Cibarian	0·13	1·79	4·36
Karakin	1·23	2·79	6·34
Hiptagin	trace	1·67	4·11
3-Nitropropanol	0·07	2·10	4·74
3-Nitropropanoic acid	0·65	4·00	8·90

* HPLC pump pressures were 1600–1800 psi and the flow rate was 3·0 ml/min.
† The amount of each compound present was determined by comparison of UV peak areas with standard samples.

A 220 Hz NMR of our karakin sample and proton assignments (see Experimental) confirmed the 1,2,6-tri-(3-nitropropanyl)-1-β-D-glucopyranoside structure. The ratio of content of karakin to the 1,6-diester (cibarian) was about 2:1 in *A. canadensis* and about 5:1 in *A. flexuosus*.

Constituents of *A. flexuosus* were further studied by TLC and HPLC methods of analysis. TLC was used to separate constituents of a crude plant extract and the nitro containing compounds present were visualized with a Griess–Ilosvay type spray. Table 1 contains a list of compounds identified from *A. flexuosus* and the R_f values in the two solvent systems used. Each nitro-positive spot on the TLC plate was checked by comparison with known samples by checking R_f values as well as by isolation through preparative TLC and subsequent NMR analysis.

The second chromatographic method used HPLC on a micro-Porasil column with two different solvent systems and UV detection at 254 nm. From the area of the peaks recorded by UV detection a determination of the amount present of each nitro-containing compound could be made (Table 2).

Both of the above chromatographic methods present new potential for the separation and identification of the nitro-containing compounds in crude plant extracts and are now being used for studies of a number of different species of *Astragalus* that are known to cause locoweed poisoning [8] or have been suspected of containing toxic nitro compounds.

The presence of the highly toxic components of miserotoxin and 3-nitropropanol in minor amounts in *A. flexuosus* is of particular interest. These were previously reported only from *A. miser* and were not reported from *A. cibarius*. It seems likely that these compounds (and perhaps the whole range of nitropropanoic acid glucosides) will be found present in most if not all of the nitro-containing *Astragalus* species, with individual species variations limited to changes in relative amounts. Preliminary work with several other species has tended to strengthen this hypothesis.

EXPERIMENTAL

Isolation of major components. Astragalus canadensis var. *Mortonii* (Nutt.) Wats. was collected in August 1972 at Bass Creek (Missoula Co.), Montana (Colorado State University Herbarium Accession No. 52310) and *A. flexuosus* var. *flexuosus* (Hook) Don was collected in June 1972 at Horsetooth Reservoir (Larimer Co.), Colorado (C.S.U. Herbarium Accession No. 51718). Whole above-ground plant material of *Astragalus canadensis* var. *Mortonii* and *Astragalus flexuosus* var. *flexuosus* was air-dried and pulverized to 60 mesh. The ground plant

material (0·33 kg) was extracted with 2·5 l. acetone over 24 hr with stirring. The Me_2CO was evaporated to yield a thick green tar which was extracted with (0·3 l.) hexane and the solvent evaporated to yield a crude brown residue. *A. canadensis* yielded 15·2 g/kg and *A. flexuosus* 27·5 g/kg of crude residue. Each residue was dissolved in Me_2CO, adsorbed on to 30 g of silica gel, dried gently (N_2) and chromatographed on a column of 1·8 kg silica gel being successively eluted with 500 ml mixtures of Me_2CO in $CHCl_3$ (0–80%). Each fraction was collected, dried, and 100 MHz NMR spectrum were run on every 4th fraction redissolved in d_6-C_4H_{10}. Each fraction was also tested with the Griess–Ilosvay reagent [9] to determine if nitro-containing compounds were present. Fractions 21–31 yielded 1,6-di-(3-nitropropanoyl)-β-D-glucopyranoside (cibarian) (100 MHz NMR, m.m.p. and optical rotation with a standard sample). Fractions 103–136 gave karakin recrystallized from MeOH–$CHCl_3$ and then MeOH–isooctane, m.p. 124–125° [3] m.p. 123·5–125° for karakin). The 220 MHz NMR (Morgan–Schaffer Corp., d_6-acetone) was analyzed as follows (δ values in p.p.m. from TMS); 3·05–3·14 (broad triplet, 6H, $-OOCC\underline{H}_2CH_2NO_2$); 3·25 (*t*, 1H, proton at C-4 coupled to C_3 and C_5, J_{34} 9·5, J_{45} 10); 3·75–3·85 (*m*, 2H, protons at C-3 and C-5); 4·42 (*m* of eight peaks, 2H, protons at C-6 and C-6', $J_{66'}$ 12·5, J_{56} 5·5, $J_{56'}$ 2·4); 4·76 (*t*, 2H, $-OOCCH_2C\underline{H}_2NO_2$, *J* 5·8); 4·81 (*t*, 2H, $-OOCCH_2C\underline{H}_2NO_2$, *J* 5·8); 4·87 (*t*, 2H, $-OOCCH_2C\underline{H}_2NO$, *J* 5·8); 5·78 (*d*, 1H, *J* 8·4, anomeric proton C-1). M.S. (CH_4) 466 loss of H_2O from MH^+ (484), 365, 318, 256, 199, 174 *m/e*.

*Thin layer chromatography.** *A. flexuosus* crude extract in Me_2CO was separated on silica gel G, (250 μ) using the solvents in Table 1. The plates were sprayed with 20% NaOH, dried 5 min at 130°, and then resprayed with Griess–Ilosvay reagent and again dried 5 min at 130°. Finally the plates were lightly sprayed with 2 N HOAc and redried at 130°. Pink–red spots were obtained for nitro compounds. The preparative TLC was on pre-coated TLC plates coated silica gel F-254, (2 mm) developed in C_4H_{10}–$CHCl_3$ (1 : 1). The bands of nitro-containing compounds were detected in a test strip, the components extracted with acetone, and their identity confirmed by NMR.

* Another TLC identification method was reported recently: Majak, W. and Bose, R. J. (1974) *Phytochemistry* **13**, 1005.

High pressure liquid chromatography. All HPLC investigations were carried out on an HPLC model ALC/GPC 301 from Waters Associates, Inc. with UV detection at 254 nm. Samples were prepared by taking 50–100 mg of crude *A. flexuosus* extract dissolved in 2 ml of reagent grade acetonitrile. Injections were 2–10 μl of the acetonitrile mixture. Columns were 6mm × 30 cm packed with micro-Porasil.

Periodate oxidations. (a) 50 mg of the karakin sample in to 4 ml of acetate buffer (pH 4·0) containing 1·5 g of $NaIO_4$ was diluted to 5 ml and changes in the optical rotation were determined up to 48 hr [10]. (b) 12 mg of karakin in 50 ml of acetate buffer (pH 4·0) and 2·0 ml of 0·346 M $NaIO_4$ was added. Aliquots were removed, diluted 1 to 100 and the absorbance was measured at 270 nm. Readings were taken up to 48 hr. Karakin consumed 0·68 mol of periodate in 6 hr; 1·14 mol in 12 hr; 1·52 mol in 18 hr; 1·68 mol in 24 hr and 1·78 mol in 30 hr.

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