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# NITROPROPANYLGLUCOPYRANOSES IN *CORONILLA VARIA*

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**Key Word Index**—*Coronilla varia*; Leguminosae; crownvetch; *Astragalus* spp; aliphatic nitro compounds; nitropropanylglucopyranoses.

**Abstract**—The isolation and characterization of 6-(3-nitropropanyl)-D-glucopyranose from *Coronilla varia* is described and the concentration of the compound is determined by quantitative microisolation. Di- and tri-nitropropanylglucopyranoses are detected in *C. varia* and three *Astragalus* spp.

## INTRODUCTION

The suitability of *Coronilla varia* L. (crownvetch) for erosion control of highway embankments and disturbed slopes has been demonstrated in the Eastern United States [1,2]. The advisability of widespread seeding of crownvetch, however, is questionable in the light of recent toxicity studies with meadow voles [3,4] which identified 3-nitropropionic acid (NPA) as a toxic factor in *C. varia* cv. Penngift [5]. NPA, di-, tri- and tetra-substituted glucose esters of NPA were described by others as the toxic constituents of *Indigofera endecaphylla* [6–10]. Earlier workers showed that ingestion of *I. endecaphylla* was lethal to rabbits and chickens and caused abortions or stillbirths in pregnant cows and heifers [11,12]. We detected bound forms of NPA in *C. varia* and this study describes the isolation and characterization of 3-nitropropanylglucopyranoses in crownvetch.

## RESULTS AND DISCUSSION

The nitropropanylglucopyranoses were purified by PC and PLC as described in the Experimental. The use of mercaptoethanol in the first solvent was necessary to prevent degradation of the nitro compounds during PC. Extraction of fresh or fresh-frozen plant material was preferred since parallel extractions of oven-dried (55°, 48 hr) samples of crownvetch showed a 30–40% reduction in the concentration of NPA conjugates as determined by quantitative microisolations.

### 6-(3-*nitropropanyl*)-D-*glucopyranose* (1)

The compound, isolated from the ethyl acetate extract of crownvetch, gave a positive test for reducing sugars (*p*-anisidine phthalate reagent [13]), and yielded the specific colour changes of primary aliphatic nitro compounds after spraying with the diazotized *p*-nitroaniline reagent (System A [14]). The $R_f$'s for **1** on Avicel PLC in BEW, BAW, Pyr and PrOH (the key to the solvent systems has been explained previously [14]) were 0·43, 0·50, 0·57 and 0·61 respectively as compared with cibarian (1,6-di-(3-nitropropanyl)-$\beta$-D-glucopyranose [15]) which yielded $R_f$ values of 0·63, 0·60, 0·76, and 0·73 respectively, and karakin (1,4,6-tri-(3-nitropropanyl)-$\beta$-D-glucopyranose [6]) which gave $R_f$ values of 0·77, 0·71, 0·89 and 0·81 respectively. A relationship was evident between substitution pattern and $R_f$. Acid or alkaline hydrolysis of **1** yielded glucose and NPA which were identified by specific spray reagents on PC and TLC as described previously [14]. The elemental analysis of **1** was consistent with a mono-3-nitropropanylhexose. Partial acid hydrolysis of cibarian yielded glucose, NPA and a third hydrolysis product which gave a positive test for reducing sugars, a positive test for primary aliphatic nitro compounds and co-chromatographed with the isolated **1** in five solvents.

The 100 MHz NMR spectrum of **1** (4 times exchanged with 99·96% $D_2O$) was in accord with the proposed structure. Poorly resolved triplets at $\delta$ 3·58 and $\delta$ 5·26 ppm from external TMS corresponded to the acyl and nitro methylene protons of the 3-nitropropanyl substituent respectively. A sharp doublet centered at $\delta$ 5·63 ppm, with a coupling constant of 3 Hz, arises from the anomeric proton of the $\alpha$-D-glucopyranose residue and was non-integral (0·5 H) with respect to the nitropropanyl substituent. However, integration of all protons and comparison with the integral from the 3-nitropropanyl substituent established **1** to be monosubstituted. The poor resolution of the pair of triplets assigned to the 3-nitropropanyl group occurs, presumably, because the spectrum is that of an approximate 1:1 mixture of the $\alpha$ and $\beta$ anomers of 6-(3-nitropropanyl)-D-glucopyranose, the anomeric proton of the $\beta$ anomer being obscured by other high field protons. The NMR of the TMSi

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derivative of **1** gives a poorly resolved triplet for the nitro methylene protons centered at $\delta$ 4·4 ppm and a multiplet (overlapping triplets) centered at $\delta$ 2·79 ppm for the acyl methylene protons. Irradiation of the low field triplet effected a collapse of the acyl methylene protons to a pair of closely spaced ($\Delta\upsilon = 2$ Hz) singlets of about equal intensity, reflecting the small chemical shift differences which exist for the acyl methylene protons of the 3-nitropropanyl substituent at $C_6$ in the two anomeric forms. By contrast irradiation of the nitro methylene proton multiplet in cibarian effected a collapse of the acyl methylene multiplet to a pair of singlets with a shift difference of 8 Hz [16] consistent with what could be expected when the substituents occur in two distinctly different positions in the hexose residue.

While it was not possible to assign the position of the 3-nitropropanyl group on the glucose moiety from the complex NMR spectrum, the mass spectrum of the TMSi derivative of **1** provides convincing evidence in support of the proposed structure. The molecular ion (*m/e* 569) is readily apparent in the spectrum as the highest mass ion with an intensity 40% of that of the characteristic M-15 ion (*m/e* 554) normally observed as the highest mass fragmentation in the spectra of TMSi ethers of monosaccharides [17]. Several relatively intense ions occur in the high mass region of the spectrum of TMSi-**1** exactly 29 mass units above the analogous ions present in the mass spectrum [17] of 1,2,3,4,6-pentakis-*O*-(trimethylsilyl)-α-D-glucopyranoside (TMSi–glucopyranoside); 29 mass units being the difference between the TMSi and 3-nitropropanyl substituents. Thus, fragment ions occur at *m/e* 554, 464 and 374 and correspond to the loss of 15(Me·), 105 (Me· and TMSiOH) and 195 (Me· and 2 TMSiOH) mass units respectively. The ion fragment *m/e* 435 in the mass spectrum of TMSi-**1** also occurs in the mass spectrum of the TMSi-hexoses, and presumably corresponds to the loss of nitropropionic acid from the ion-fragment generated by the loss of the methyl radical from the molecular ion-radical. The 6-position of the 3-nitropropanyl substituent is established from the intense ions at *m/e* 217 (38%), 204 (91%) and 191 (32%) relative to the most intense ion at *m/e* 73 (100%) for the $TMSi^+$ ion, and for which the structures $TMSi\overset{+}{O}{=}CH{-}CH{=}CH{-}OTMSi$, $[TMSiO{-}CH{=}CH{-}\overset{+}{O}{-}TMSi]^+$, and $TMSiOCH{=}\overset{+}{O}{-}TMSi$ respectively have been assigned by exact mass measurement [18] and deuterium labelling has shown these ion fragments to originate predominantly from $C_{2,3,4}$, $C_{2,3}$ and $C_1$ carbons of the hexose skeleton respectively [18]. That these ions constitute the most intense ions, apart from *m/e* 73, in the mass spectrum of TMSi-**1** and in the approximate relative abundancies as occurs in the mass spectrum of TMSi-glucopyranoside, strongly suggests TMSi-**1** bears TMSi ethers on carbons 1, 2, 3 and 4 and therefore the 3-nitropropanyl group on carbon 6.

*Di- and tri-nitropropanylglucopyranoses*

The presence of an overlapping 3-nitropropanylglucopyranose (see below) necessitated three chromatographic developments (in BEW, on long TLC plates) to achieve purification of cibarian, isolated from the ether extract of crownvetch. The cibarian isolate gave an NMR identical to an authentic sample of cibarian (courtesy Prof. F. R. Stermitz) and co-chromatographed with the original standard in five solvents.

Quantitative microisolations showed that the concentrations of cibarian and **1** were 2·6 and 2·0% (dry wt) respectively in fresh prebloom aerial shoots of crownvetch and only traces of NPA could be detected. Likewise, Gustine *et al.* reported that their NPA isolate accounted for less than 10% of the toxic fraction in crownvetch, and they proposed that the remaining toxic components were NPA glucose esters [5]. We also detected chromatographically cibarian and **1** in field samples of *Astragalus canadensis* L., *A. collinus* Dougl., and *A. robbinsii* (Oakes) Gray which occur sporadically throughout south-central British Columbia [19].

In addition to the above, crownvetch yielded minor amounts of an NPA ester ($R_f$ 0·68 in BEW) which overlapped with cibarian ($R_f$ 0·63 in BEW) in a variety of solvents. Although repeated chromatographic developments were required for preparative isolations, resolution during microisolations was achieved on a single Avicel plate (20 × 40 cm).

Partial acid hydrolysis of this derivative yielded glucose, NPA, **1**, and a second reducing nitropropanylglucopyranose ($R_f$ 0·47 in BEW), which migrated adjacent to **1** ($R_f$ 0·43 in BEW). The results would indicate that this compound is a reducing NPA diester of glucose with one of the nitropropanyl substituents in the 6 position. The NMR spectrum of this isolate in $D_2O$ (thrice exchanged with $D_2O$), although complex, gave an integral for the 3-nitropropanyl protons with an intensity relative to the anomeric and other protons, which corresponded exactly to the presence of two such residues per hexose unit. As in **1**, the NMR of this material showed a sharp doublet ($\delta$ 5·73 ppm) with a $J$ value of 3 Hz and an intensity corresponding to approximately 0·4 protons; the remaining absorbance from $H_1$, presumably the $\beta$ anomer, was obscured by other high field protons in the spectrum, as was the case for **1**. Finnegan has reported [15] substitution patterns of 4,6-α, 4,6-β, and 2,6-α for NPA diesters of *Indigofera endecaphylla* and our isolate may be similar. Karakin was also detected in the field samples of crownvetch as evidenced by co-chromatography with an authentic sample of karakin (courtesy Prof. F. R. Stermitz) in five solvents.

## EXPERIMENTAL

*Plant material.* Aerial shoots were harvested from a mature stand of *Coronilla varia* L. cv. Penngift from the nursery at Lethbridge Research Station, Agriculture Canada, Alberta, and this material, fresh-frozen, was used for large-scale extractions. Microisolations were performed with fresh material from a new stand (seeded in 1975) of *C. varia* cv. Penngift established under growth room conditions at Kamloops. *Astragalus* spp. were collected near Kamloops, B.C. in 1974 and voucher specimens were deposited in the Research Station herbarium, Agriculture Canada, Kamloops.

*Large scale isolation.* Plant material (1·3 kg) was extracted with 12 l. 80% EtOH in a Waring blender and the filtrate treated with Celite and polyamide [14]. The polyamide eluate was concentrated and extracted with $Et_2O$ for 48 hr in a continuous extractor and the remaining phase re-extracted continuously with EtOAc for 48 hr. During chromatography aliphatic nitro compounds were detected with the diazotized *p*-nitroaniline spray reagent and with UV light [14]. Avicel was washed prior to spreading [14], and layers were 1 mm thick. The $Et_2O$ extract was chromatographed successively in the following systems: BEW containing 0·1% mercaptoethanol on Whatman no. 3 MM chromatography paper and 3× in BEW on Avicel PLC plates (20 × 56 cm). The EtOAc extract, which

yielded **1**, was chromatographed successively in the following solvent systems: BEW-mercaptoethanol (as above); BEW (Avicel 20 × 40 cm); EtOH–$H_2O$, 1:4 (Avicel, 20 × 20 cm) and BEW (in the final step Avicel plates were initially developed in $H_2O$ and dried prior to sample application). The final eluates were concentrated and freeze-dried over $P_2O_5$ to yield white powders. Partial acid hydrolysis was carried out in N HCl over a period of 20 min at 60°. Alkaline hydrolysis was carried out in N NaOH for 30 min at room temp.

6-(3-*nitropropanyl*)-D-*glucopyranose* (**1**). Anal. found: C, 38·2; H, 5·42; N, 4·64. $C_9H_{15}NO_9$ requires: C, 38·4; H, 5·38; N, 4·98%. The IR spectrum (KBr) showed absorptions at 3400 (hydroxyl), 1735 (ester carbonyl), and 1560 $cm^{-1}$ (nitro group).

*Microisolations and quantitative determinations.* Extract 1 (from 15 g fr. wt crownvetch) was prepared as described previously [20], evaporated to dryness, redissolved in 50 ml 65% EtOH and duplicate 0·10 ml aliquots were applied to 20 × 40 cm Avicel PLC plates with adjacent standards. The plates were developed × 2 in $Me_2CO$–$H_2O$ (9:1) to a height of 8·5 cm, the bottom 6 × 20 cm layer was removed (to eliminate interfering flavonoids) and development proceeded overnight in BEW. The cibarian and **1** bands were eluted with $H_2O$ and coupled in alkaline solution with diazotized *p*-nitroaniline prior to colorimetric determination as described for miserotoxin [14]. The alkali lability of NPA esters, however, necessitated the immediate addition of the diazotized *p*-nitroaniline reagent following the introduction of NaOH to the eluates. The diazotized *p*-nitroaniline reagent, prepared by combining 6·5 ml 2·5% $NaNO_2$ (in $H_2O$) with 100 ml 0·3% *p*-nitroaniline (in N HCl) was stable for 3 months if stored in the dark at 4°. Standard curves for **1** (1–10 ppm) and cibarian (1–7 ppm) were prepared from freeze-dried isolates.

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