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NITROALIPHATIC COMPOUNDS IN *HIPPOCREPIS COMOSA* AND OTHER LEGUMES IN THE EUROPEAN FLORA

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Key Words Index—*Hippocrepis comosa*; *Lotus uliginosus*; Fabaceae; Leguminosae; horseshoe vetch; nitropropionate.

Abstract—In a survey of 148, mostly European, angiosperm species, 3-nitropropionate was found only in extracts of the legumes, *Hippocrepis balearica*, *H. comosa*, *Lotus uliginosus* and *Scorpiurus muricatus*. Three separate nitropropanoyl esters of glucose, 6-(3-nitropropanoyl)-α/β-D-glucopyranose, 1,6-di-*O*-(3-nitropropanoyl-β-D-glucopyranose (cibarian) and 1,2,6-tri-*O*-(3-nitropropanoyl)-β-D-glucopyranose (karakin) were identified in shoot extracts of *H. comosa*. Evidence for the presence of the 3-nitropropanoyl ester of 4-hydroxybutanoic acid in extracts of *H. comosa* is also presented.

INTRODUCTION

The occurrence of nitroaliphatic compounds in angiosperms is well documented [1] and although screening indicates that these compounds are not widely distributed in nature [2] they have been found in a number of leguminous (Fabaceae) plants, notably *Securigera* (*Coronilla*) *varia* [3–6] *Indigofera* spp. [7, 8] *Astragalus* spp. [9–16] and in certain members of the Corynocarpaceae [17] and Malpighiaceae [2]. However, most of these studies have concentrated on American and southern hemisphere floras and there has been little investigation of the occurrence of nitroaliphatics in European species. Since nitroaliphatic compounds such as 3-nitropropanoic acid (3NPA) are known to be toxic to livestock and other animals [1], knowledge of their distribution in European pasture plants is desirable. Moreover, little is known of the taxonomic or ecological significance of these compounds in plants.

RESULTS AND DISCUSSION

Survey of the occurrence of 3-nitropropionate and nitrite in shoot extracts

Tables 1 and 2 show the results of a survey of 65 species in the Fabaceae for the presence of 3NPA and/or nitrite in shoot, cell-free extracts. High levels of 3NPA ($> 10\ \mu mol\ g^{-1}$ fr. wt) were found in extracts from five leguminous species, *Securigera* (*Coronilla*) *varia*, *Hippocrepis balearica*, *H. comosa*, *Lotus uliginosus* and *Scorpiurus muricatus* (Table 1). Extracts from these plants also contained high levels of nitrite which results from the enzymatic oxidation of 3NPA. High levels of nitrite were also found in extracts from *Hippocrepis ciliata* and *Lotus purpureus*, but the presence of 3NPA in extracts from these plants was not tested. These results suggest that 3NPA is of restricted occurrence in legumes since in this survey only members of the tribes Loteae and Coronilleae were found to contain this compound. Although a number of American species in the genus *Astragalus* (Galegeae) [9–16] have been shown to contain 3NPA, 3NPA esters and related substances, neither of the *Astragalus* species tested here contained 3NPA (Table 2). Of 83 non-legume species tested, representing 50 families, none contained 3NPA in their shoots (Table 2).

Given the occurrence of *H. comosa* and *L. ulignosus* in pastures frequently used for grazing livestock in Europe, the presence of toxic 3NPA in shoots of these plants is especially significant.

Identification of 3-nitropropanoyl esters of glucose in Hippocrepis comosa

The identification of 3NPA in EtOAc-fractionated, Me_2CO extracts from *H. comosa* was confirmed by ^1H NMR. Another compound was also present in these samples and evidence for its provisional identification as the 3-nitropropanoyl ester of 4-hydroxybutanoic acid is as follows. The ^1H NMR spectrum showed signals for five CH_2 groups, one of which overlapped with the 3NPA triplet at δ4.66. A COSY measurement established the proton connectivities and indicated the presence of

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Table 1. 3NPA and nitrite in members of the Fabaceae*

	3NPA	Nitrate
Tribe — Loteae		
Anthyllis vulneraria L.	0	0
Lotus corniculatus L.	0	0
L. purpureus Moench.	ND†	36.8
L. uliginosus Schkuhr	10.1	2.6
Tribe — Coronilleae		
Coronilla emerus L.	0	0
C. scorpioides (L.) Koch	0	0
Hippocrepis balearica Jacq.	223.5	+
H. ciliata Willd.	ND	88.0
H. comosa L.	142.0	+
Ornithopus perpusillus L.	0	0
Securigera (*Coronilla*) *varia* (L.) Lassen	52.5	+
Scorpiurus muricatus L.	25.9	+

* 3NPA and nitrite are expressed as μmol g^{-1} fr. wt.
†ND, not determined.

an isolated pair of methylene groups corresponding to 3NPA, and of three contiguous methylene groups corresponding to the fragment $OCH_2CH_2CH_2COOH$. The integral indicated a ratio of 1.4/1 for these two constituents, 3NPA/3NPA-ester. A pseudo-molecular ion was detected at m/z 223 $[M + NH_4]^+$ in the CI mass spectrum and confirmed by a high-resolution measurement.

A crystalline solid derived from a 50% EtOAc fraction was shown to be 1,2,6-tri-*O*-(3-nitropropanoyl)-β-D-glucopyranose (karakin) [8] by proton and ^{13}C NMR [18]. Recrystallization gave the pure triester. A 55% EtOAc fraction was shown to be mainly 1,6,-di-*O*-(3-nitropropanoly)-β-D-glucopyranose (cibarian) [10] by ^1H NMR measurements. A 50% EtOH fraction gave a complex NMR spectrum due to the presence of both anomers of a glucose derivative. The integral showed that it was a mono-3-nitropropanoyl ester and the chemical shifts of the protons on C-6 of both anomers established that the compound was 6-*O*-(3-nitropropanoyl)-$\alpha\beta$-D-glucopyranose. This compound has been reported from *Securigera* (*Coronilla*) *varia* [4] where mass spectrometry of the

Table 2. Species in which 3NPA and nitrite were not detected (including assignment to tribes for Fabaceae)

ACERACEAE: *Acer pseudoplatinus* L.; ALISMATACEAE: *Alisma plantagoauatica* L.; APIACEAE: *Aegopodium podagraria* L.; *Oenanthe crocata* L.; AQUIFOLIACEAE; *Ilex aquifolium* L.; ARALIACEAE: *Hedera helix* L.; ARECACEAE; *Phoenix dactylifera* L.; ASTERACEAE: *Achillea millefolium* L.; *Bellis perennis* L.; *Centaurea scabiosa* L.; *Cirsium arvense* (L.) Scop.; *Eupatorium cannabinum* L.; *Petasites hybridus* (L.) Gaertner; *Pulicaria dysenterica* (L.) Bernh.; *Senecio jacobaea* L.; *S. vulgaris* L.; *Taraxacum officinale* agg. Weber; BETULACEAE: *Betula pendula* Roth.; BORAGINACEAE: *Symphytum officinale* L.; BUDDLEJACEAE: *Buddleja davidii* Franchet; CARYOPHYLLACEAE: *Moerhingia trinervia* (L.) Clairv.; *Saponaria officinalis* L.; *Stellaria media* (L.) Vill.; CHENOPODIACEAE: *Chenopodium album* L.; CISTACEAE: *Helianthemum nummularium* (L.) Miller: CLUSIACEAE: *Hypericum elodes* L.; *H. tetrapterum* Fries CONVOLVULACEAE; *Calystegia sepium* (L.) R. Br.; *Convolvulus arvensis* L.; CRASSULACEAE: *Sedum acre* L.; *S. album* L.; CYPERACEAE: *Carex montana* L.; *C. pendula* Hudson; DIPSACACEAE; *Scabiosa columbaria* L.; ELAEAGNACEAE: *Hippophae rhamnoides* L.; ERICACEAE: *Erica tetralix* L.; EUPHORBIACEAE: *Euphorbia peplus* L.; *E. portlandica* L.; FABACEAE: (Tribe — Robineae) *Robinia pseudacacia* L.; *Wisteria sinensis* (Sims) Sweet; (Tribe — Phaseoleae) *Glycine max* (L.) Merr.; *Phaseolus vulgaris* L.; *Vigna unguiculata* (L.) Walpers; (Tribe – Galegeae) *Astragalus clusii* Boiss.; *A. glycyphylos* L.; *Galega officinalis* L.; (Tribe — Hedysareae) *Hedysarum coronarium* L.; *Onobrychis viciifolia Scop.*; (Tribe — Aeschynomeneae) *Arachis hypogeae* L.; (Tribe — Fabeae) *Lathyrus latifolius* L.; *L. montanus* Bernh., *L. odoratus* L.; *L. pratensis* L.; *L. tuberosus* L.; *Lens culinaris* Medicus; *Pisum sativum* L.; *Vicia cracca* L.; *V. hirsuta* (L.) S. F. Gray; *V. orobus* D. C.; *V. sativa* L.; *V. sepium* L.; *V. sylvatica* L.; (Tribe — Cicereae) *Cicer arietinum* L.; (Tribe — Trifoleae) *Medicago arabica* (L.) Hudson; *M. officinalis* (L.) Pallas; *Ononis reclinata* L.; *O. repens* L.; *Trifolium arvense* L.; *T. campestre* Schreber; *T. dubium* Sibth.; *T. hybridum* L.; *T. medium* L.; *T. micranthum* Viv.; *T. ornithopodioides* L.; *T. pannonicum* Jacq.; *T. pratense* L.; *T. ochroleucon* Hudson; *T. repens* L.; *T. scabrum* L.; *T. striatum* L.; (Tribe — Genisteae) *Chamaespartium sagittale* (L.) P. Gibbs; *Cytisus scoparius* (L.) Link; *Genista anglica* L.; *G. tinctoria* L.; *Laburnum anagyroides* Medicus; *Lupinus arboreus* L.; *Petteria ramentacea* (Sieber) C. Presl.; *Ulex europaeus* L.; FAGACEAE: *Quercus petraea* (Mattuschka) Liebl.; FUMARIACEAE: *Corydalis lutea* (L.) D. C.; *Fumaria officinalis* L.; GERANIACEAE: *Geranium robertianum* L.; *G. sanguineum* L.; GENTIANACEAE: *Centaurium erythraea* Rafn.; HIPPOCASTANACEAE: *Aesculus hippocastanum* L.; IRIDACEAE: *Iris pseudacorus* L.; JUNCACEAE: *Juncus effusus* L.; *J. tenuis* Willd.; LAMIACEAE: *Acinos arvensis* (Lam.) Dandy; *Mentha aquatica* L.; LILIACEAE: *Allium schoenoprassum* L.; *Narthecium ossifragum* (*L.*) Hudson; *Polygonatum multiflorum* (L.) All.; LYTHRACEAE: *Lythrum salicaria* L.; MALVACEAE: *Althaea officinalis* L.; *Malva sylvestris* L.; MENYANTHACEAE: *Menyanthes trifoliata* L.; *Nymphoides peltata* (S. G. Gmelin) O. Kuntze; NYMPHAEACEAE: *Nymphaea alba* L.; OLEACEAE: *Ligustrum ovalifolium* Hassk.; ONAGRACEAE: *Chamerion angustifolium* (L.) Holub; *Epilobium hirsutum* L.; PAPAVERACEAE: *Meconopsis cambrica* (L.) Vig.; PLANTAGINACEAE: *Plantago lanceolata* L.; POLYGONACEAE: *Rumex* acetosella L.; PRIMULACEAE: *Anagallis arvensis* L.; RANUNCULACEAE: *Clematis vitalba* L.; *Ranunculus flammula* L.; ROSACEAE: *Potentilla anserina* L.; *Rosa pimpinellifolia* L.; *Sangisorba minor* Scop.; RUBIACEAE: *Asperula cynanchica* L.; *Galium verum* L.; SALICACEAE: *Salix babylonica* L. SCROPHULARIACEAE: *Eurphrasia nemorosa* (Pers.) Wallr.; *Linaria vulgaris* Miller; *Odontites verna* (Bell.) Dumort.; *Verbascum thapsus* L.; SOLANACEAE: *Solanum dulcamara* L.; URTICACEAE: *Urtica dioica* L.; VALERIANACEAE: *Valeriana officinalis* L.; VERBENACEAE: *Verbena officinalis* L.; VIOLACEAE: *Viola palustris* L.; *V. riviniana* Reichenb.

trimethylsilyl ether derivitive was used to establish the structure. In our study, the 400 MHz spectrum was assigned completely with the aid of spin decoupling, and the signals of the nitropropanoyl groups in the two anomers were just resolved. The spectrum closely resembled that reported recently for the same ester isolated from *Corynocarpus laevigatus*, in a different solvent [19].

EXPERIMENTAL

General. NMR: Bruker AC400 spectrometer; EI and CIMS: VG 12-250 mass spectrometer; HRMS: VG Analytical ZAB-E fitted with a 11-250 J system.

Survey of the occurrence of 3NPA. Most plants were sampled from natural populations within the vicinity of University of Wales, Swansea or (with pulses and horticultural varieties) obtained from greenhouse collections; 3-NPA in shoot extracts from these plants was determined as described in ref. [20].

Extraction and isolation of nitropropanoyl esters. Shoot materials (500 g) from *Hippocrepis comosa* was extracted with Me_2CO or EtOH and subjected to silica gel (90–130 mesh) chromatography with a 2×100 cm or 5×60 cm column, as described before [10, 17]. 3NPA in pooled 35–65% EtOAc fractions was identified after comparison with an authentic sample using TLC [5] ^{1}H NMR and mass spectrometry; EI (probe) 70 eV; *m/z* 102 $[M-OH]^+$; CIMS (NH_3, probe): *m/z* 137 $[M + NH_4]^+$. The ^{1}H NMR spectrum also indicated the presence of 4-(3-nitropropanoyloxy)butanoic acid; δ400 MHz, ($CDCl_3$) 4.66 (*t*,$OCH_2CH_2NO_2$), 4.22 (*t*, $-OCH_2CH_2-$), 3.06 (*t*, $-O_2CH_2CH_2NO_2$ of NPA), 2.98 (*t*, $O_2CCH_2CH_2NO_2$), 2.48 (*t*, $-CH_2CH_2COOH$), 2.01 (*quintet*, $CH_2CH_2CH_2COOH$) (OH was not detected); CIMS (NH_3, probe): *m/z* 223 $[M + NH_4]^+$, found 223.0930, $C_7H_{15}N_2O_6$ requires 223.0930; EIMS (probe) 70 eV: *m/z* 206 $[M + 1]^+$, 188 $[M - OH]^+$.

Karakin was isolated as a crystalline solid; mp 121–123° after recrystallization from MeOH–$CHCl_3$ (lit. 122–123 C [17], 124–125°C [12]); ^{1}H and ^{13}C NMR: identical to literature data [8]; MS (FAB) *m/z* 506 $[M + Na]^+$. Cibarian was isolated as a syrup; ^{1}H NMR: identical with literature data allowing for the external TMS reference used [10].

6-*O*-(3-Nitropropanoyl)-α-β-D-glucopyranose was isolated as a syrup; ^{1}H NMR (400 MHz d_4-MeOH): δ5.10 (*d*, $J = 3.7$ Hz, H-1α), 4.738 (*t*, $J = 6.0$ Hz, CH_2NO_2), 4.736 (*t*, $J = 5.8$ Hz, CH_2NO_2), 4.65 (*bs*, OH), 4.51 (*d*, $J = 7.9$ Hz, H-1β), 4.66 (*dd*, $J = 2.2$ and 11.8 Hz, H-6β), 4.40 (*dd*, $J = 2.2$ and 11.8 Hz, H-6α), 4.27 (*dd*, $J = 5.3$ and 11.8 Hz, H-6′α), 4.24 (*dd*, $J = 5.9$ and 11.9 Hz, H-6′β), 3.98 (*oct*, H-5α), 3.68 (*t*, $J = 9.3$ Hz, H-3α), 3.51 (*oct*, H-5β), 3.39 (*dd*, $J = 3.7$ and 9.6 Hz, H-2α), 3.38 (*t*, $J = 8.9$ and 9.1 Hz, H-3β), 3.32 (*t*, $J = 9.7$ Hz, H-4β), 3.31 (*t*, $J = 9.5$ Hz, H-4α), 3.16 (*dd*, $J = 7.9$ and 9.1 Hz, H-2β), 3.048 (*t*, $J = 5.8$ Hz, CH_2CO), 3.046 (*t*, $J = 6.0$ Hz, CH_2CO) (intensities of α and β signals were approximately equal); MS (FAB: *m/z* 304 $[M + Na]^+$.

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