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ISOVITEXIN 6″-*O*-β-D-GLUCOPYRANOSIDE: A FEEDING DETERRENT TO *PIERIS NAPI OLERACEA* FROM *ALLIARIA PETIOLATA*

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Key Word Index—*Alliaria petiolata*; Cruciferae; *Pieris napi oleracea*; Pieridae; flavone glycoside; feeding deterrent.

Abstract—*Pieris napi oleracea*, an indigenous butterfly to North America lays eggs on *Alliaria petiolata*, an invasive weed introduced from Europe, but the larvae generally do not survive. A new apigenin glycoside, isovitexin 6″-*O*-glucoside has been isolated from the leaves *A. petiolata* and identified as a feeding deterrent for *P. napi* fourth instar larvae. The structure was elucidated by UV, MS and NMR spectroscopy. © 1998 Published by Elsevier Science Ltd. All rights reserved

INTRODUCTION

Alliaria petiolata (Garlic mustard) is an exotic plant species that was introduced to North America from Europe more than 130 years ago [1], and that has invaded woodlands and degraded habitats in several parts of the midwestern and northeastern United States and Canada. Because of its aggressive biology, it is displacing natural understory crucifers and other flora, and its elimination from most areas is virtually impossible [2]. Chew *et al.* (1995) have shown that *Pieris napi oleracea*, an indigenous butterfly species in the northeastern U.S.A., lays eggs on many palearctic crucifer species, including *Alliaria petiolata*. But on this species either the larvae do not survive or they vary considerably in their development. Secondary metabolites of these plants are undoubtedly involved in the interference with development [3]. Therefore, we investigated the chemical constituents of *Alliaria petiolata* that affect the normal growth of *P. napi oleracea* larvae. Here we present results of bioassay-guided isolation and identification of a new apigenin glycoside (**1**) that acts as a feeding deterrent to 4th instar larvae of *P. napi oleracea*.

RESULTS AND DISCUSSION

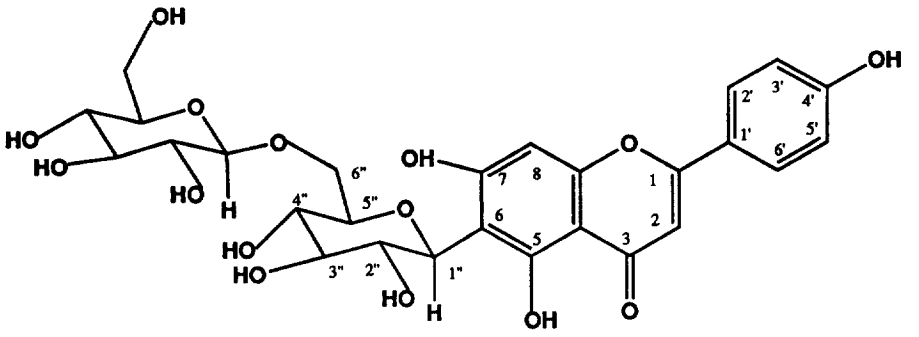
Isolation of active compounds

Isolation of feeding deterrents was monitored by behavioral bioassays (see Experimental). An ethanolic extract of *Alliaria petiolata*, was separated into hexane, chloroform, ethyl acetate, and *n*-butanol fractions by solvent extraction. The activity was found primarily in the *n*-butanol extract. The *n*-butanol extract was further fractionated on a HPLC C_{18} reverse phase column (Bondex C18, Phenomenex), monitored by a photodiode array detector (PDA) at 218 nm and 254 nm. Fractions F1–4 were separated on the basis of the HPLC pattern. Fractions F2 and F3 contained mainly apigenin flavonoids and F4 a kaempferol derivative, based on their UV spectra.

The total *n*-butanol extract and fractions F2–F4 were bioassayed for feeding deterrent activity. The *n*-butanol fraction and F2 were significantly active (FDI 40.6 and 48.4, respectively) to fourth instar larvae of *P. n. oleracea*, whereas other fractions did not inhibit feeding (Table 1); also F2 did not inhibit feeding by neonate larvae (Zhang and Renwick unpublished results). F2 contained a single compound which was characterized as an apigenin flavonoid on the basis of its UV absorbance pattern. This compound was further purified by HPLC to obtain pure **1**.

Identification of **1**

The UV absorption spectrum of **1** was typical of a flavone which could be further characterized as an apigenin glycoside (λ_{max} in MeOH at 270 and 333 nm). UV shift reagent studies along with $AlCl_3$ and HCl indicated that the 5-hydroxy position is free. Shifts with NaOAc suggested that the 7-OH was not free. FAB-MS gave an $M^+ + Na$ at 617 indicating a M_r 594 for a diglycoside of apigenin. The ^1H NMR spectrum showed the presence of 6 protons in the aromatic region. Peaks at δ 7.94 and 6.91 (*d*, $J = 8.5$ Hz, 2H



1

Table 1. Responses of *P. napi oleracea* fourth instar larvae to *A. petiolata* extracts (*n*-butanol extract and F2–F4; compound **1** was in F2) in feeding deterrent bioassays

	Mean (var) area consumed (cm^2)			
Treatment	treatment	control	FDI	*P*-value
Butanol extract	0.231 (0.03)	0.419 (0.11)	40.6	0.05
F2	0.251 (0.01)	0.567 (0.01)	48.4	0.005
F3	0.459 (0.31)	0.252 (0.02)	−23.8	0.14
F4	0.193 (0.01)	0.159 (0.07)	0.08	0.33

Feeding Deterrent Index (FDI) calculated as FDI = (C−T)/(C+T) × 100 where C = average area consumed of control discs; T = Average area consumed of treated discs. *t*-test for paired means was performed to test null hypothesis.

each) were assigned to para substituted protons on ring A of the apigenin. Two singlets at δ 6.9 and 6.6 were indicative of a *C*-glycosylated flavonoid with glycosylation on ring B. A signal at δ 6.66 for a proton attached to a carbon atom observed at δ 93.6 was assigned to C-8, as the observed shifts were typical of those for free C-8 reported in the range δ 6.50–6.66 and 93–95 for protons and carbons respectively [4]. The proton attached to unsubstituted C-6 appears in the range of δ 6.2 to 6.4 and carbons δ 103 to 105. The peak at δ 6.66 suggested that **1** is not glycosylated at the 7-*O* position, as the C-6 protons are shifted downfield by 0.3 to 0.4 ppm and carbons are shifted downfield by δ 2 to 7 ppm in case of 7-*O* substituted compounds. Finally, signals at δ 6.8 for protons attached to carbon atom δ 101.8 were assigned to C-3 [4, 5]. Two anomeric protons were observed at δ 4.64 (*d*, J = 12 Hz) attached to carbon δ 72.2 and δ 4.98 (*d*, J = 7 Hz) attached to carbon at δ 100.5, confirming that one sugar moiety is C-glycosyl and the other is *O*-glycosyl. The large coupling constants of 12 and 7 Hz suggest that both sugars are in the β-pyranosyl form.

Hydrolysis of **1** yielded a single sugar, which was identified as glucose by comparing PC patterns and GC retention times of TMS derivatives with those of authentic samples. This suggested that **1** is an *O*-glucosyl derivative of an apigenin C-6-glycoside. UV shifts for NaOAc and H_3BO_3 suggested that 7-OH is substituted, but the NMR shifts indicated that the 7-OH is free. Final unambiguous assignments and structure elucidation were possible by 2D NMR (GQCOSY, GHMQC and HMBC correlation spectra). The HMBC spectrum correlation indicated that the signal at δ 72.2 is for a carbon attached to aromatic protons and the signal at δ 100.5 was not correlated with aromatic protons. These data confirmed that the second sugar molecule forms an *O*-substituted glucoside. The C″-6 sugar carbon (—CH_2—) appeared at δ 68.5 ppm, indicating that the second sugar was substituted at the C″-6-*O*-position of the C-glycosyl moiety. This was corroborated by the HMBC correlation spectrum. The sugar attached to C-6 was identified as β-D-glucopyranosyl (C-1″ at δ 72.2 and anomeric proton at δ 4.64, J = 12 Hz) on the basis of 1D and 2D NMR evidence and by comparison with reported values, particularly with those of a luteolin derivative, isoorientin 6″-*O*-D-glucopyranoside from *Gentiana pedicellata* (Table 2) [4, 6]. J values for all sugar protons could not be calculated as there was considerable overlap of hydroxy protons. Also, ^{13}C NMR values reported for saponarin, a 7-*O*-glu-

Table 2. ^{1}H NMR and ^{13}C NMR spectral data of **1** (in DMSO-d_6, δ-values)

Carbon	^{13}C NMR **1**	^{1}H NMR	^{13}C NMR Luteolin der. 2*	^{13}C NMR Saponarin 3*
2	165.2		163.6	164.4
2	101.2	6.80 *s* 1H	103.2	103.4
4	181.7		181.8	182.3
5	156.3		156.1	156.2
6	104.7		108.7	110.7
7	162.1		163.6	162.6
8	93.6	6.66 *s* 1H	93.5	94.0
9	162.1		160.6	161.5
10	104.7		102.7	105.2
1′	128.6		121.3	121.1
2′	128.6	7.94 *d*, J = 8.5 Hz, 2H	113.2	128.8
3′	115.6	6.91 *d*, J = 8.5 Hz, 2H	145.7	116.3
4′	159.6		149.6	159.6
5′	115.6	6.91 *d*, J = 8.5 Hz, 2H	116.0	116.3
6′	128.6	7.94 *d*, J = 8.5 Hz, 2H	118.9	128.8
1″	72.2	4.64 *d*, J = 12 Hz, 1H	72.9	79.1
2″	69.9	3.92	70.4	74.0
3″	77.8	3.20	78.7	71.2
4″	72.2	4.60	70.0	69.8
5″	79.8	3.18	79.7	81.1
6″	68.5	3.18, 3.28	69.1	61.0
1‴	100.5	4.98 *d*, J = 7 Hz, 1H	103.1	104.8
2‴	74.5	3.36	73.2	71.2
3‴	76.0	3.54	76.2	72.2
4‴	72.2	4.50	69.9	66.6
5‴	73.1	3.30	73.4	71.2
6‴	60.1	3.48, 3.54	62.2	61.3

* Reported values (Refs 6 and 7).

cosylated isomer were very different from those for **1** [7].

Thus, we concluded that **1** is isovitexin 6″-*O* glucopyranoside, a compound that has not been previously reported. Its deterrent activity may be partially responsible for the lack of successful development of *P. napi oleracea* on garlic mustard.

EXPERIMENTAL

GCs were run on a capillary column DB-5 30 m × 0.23 mm in a HP5890 GC linked to a HP 5970 MSD. All ^{1}H NMR spectra were recorded at 499.99 MHz and ^{13}C NMR at 25.59 MHz.

Plants

The young rosette leaves of *Alliaria petiolata* were collected in a wooded park in Ithaca NY. Cabbage (*Brassica oleracea* L Var. Golden acre) for rearing larvae was grown in an air-conditioned greenhouse with supplemental lighting.

Insects

The larvae of *P. napi oleracea* were obtained from a continuous culture maintained in our laboratory. Only fourth instar larvae reared on cabbage were used in the bioassays.

Bioassays and analysis

Four 1.5 cm diameter cabbage leaf discs were arranged in 7 cm. diameter ice cream cups lined with moist filter paper to maintain moisture and to keep discs fresh. Test extracts 0.5 gram leaf equivalents (gle) were applied to two each of opposing discs and the other two control discs were treated with solvent alone. Four fourth instar larvae were introduced at the center of each cup, which was covered with a plastic lid. Cups were kept in an incubator at 28° for 8 h, when the remaining area of the discs was measured using a LiCor area measurement instrument. Five to 12 replications for each bioassay were performed.

Deterrent activities were compared by calculating a Feeding Deterrent Index (FDI) on the basis of area consumed. $FDI = (C-T)/(C+T) \times 100$ where C = average area consumed of control discs; T = Average area consumed of treated discs.

Extraction and isolation

Fresh young terminal and rosette leaves of *A. petiolata* were extracted with 95% ethanol (4 ml/g). The

ethanolic extract was evaporated to almost dryness *in vacuo* and the resulting mixture was partitioned successively between H_2O and hexane, chloroform, ethyl acetate and *n*-butanol. The *n*-butanol fraction was further separated by HPLC into four major fractions F1–F4. Final purification of fraction F2 was carried out by repeated HPLC.

Isovitexin-6″-O-β-D-glucoside (**1**)

FAB MS: $M^+ + Na$, 617; $M^+ + H$, 595; molecular formula $C_{27}H_{30}O_{15}$. UV λ_{max} nm: 270, and 331 (MeOH); 395, 325sh, 305sh, 270, (MeOH + NaOMe); 421sh, 390, 338, 318, 255sh, (MeOH + $AlCl_3$); 421sh, 382sh, 318, 255sh (MeOH + $AlCl_3$ + HCl); 390, 340sh, 268.8 (MeOH + NaOAc); 330, 268, (MeOH + NaOAc + H_3BO_3). ^1H NMR (499.99 MHz DMSO-d_6): and ^{13}C NMR (25.51 MHz DMSO-d_6): (Table 1).

Acid hydrolysis of **1**

Compound **1** was hydrolyzed with 2 N HCl for 4 h at 80°. The reaction mixture was extracted with ethyl acetate. The ethyl acetate fraction gave an aglycone which was confirmed as isovitexin by co-TLC. The aq. fraction was evaporated to dryness and the TMS derivative was chromatographed on GC and compared with TMS derivatives of standard sugars. On the basis of retention times the hydrolysis product was identified as glucose. The aqueous fraction was also co-chromatographed with standard sugars on paper using C_6H_6–C_5H_5N–HOAc–H_2O (5:1:3:3) and *n*-butanol–HOAc–H_2O (4:1:5) solvent systems and aniline hydrogen phthalate was used to visualize the sugars.

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Note added in proof

After acceptance of this paper for publication, a report of identification of this compound from another source *Gentiana arisanensis* (Lin, C., Kuo, S. and Chung M., *J. Nat. Prod.*, **60**, 851) has been described.

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